PATENT

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

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| Applicants: | Neeper, et al. | |
| Serial No.: | 09/642,405 - Case No.: 20413Y | Art Unit: 1632 |
| Filed: | August 21, 2000 | Examiner: |
| For: | SYNTHETIC HUMAN PAPILLOMAVIRUS GENES | Li, Q. J. |

Assistant Commissioner for Patents
BOX: Appeal Briefs-Patents
Alexandria, VA 22313

**APPEAL BRIEF**

CERTIFICATE OF MAILING

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to the: Commissioner For Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

| Denise K Brown | | 2-22-2005 |
|---|---|---|
| Name | Signature | Date |

TABLE OF CONTENTS

## REAL PARTY IN INTEREST

The real party in interest is:

Merck & Co., Inc.
One Merck Drive
Whitehouse Station, New Jersey 08889

Merck & Co., Inc. is the assignee of the entire right, title, and interest in U.S. Patent Application Serial No. 09/642,405, the subject application of this appeal.

## RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

Continuation Application Serial No. 10/728,131, filed December 4, 2003, claims priority from the present application on appeal, which claims priority from U.S. Provisional Patent Application No. 60/150,728, filed August 25, 1999, and U.S. Provisional Patent Application No. 60/210,143, filed June 7, 2000. However, Application 10/728,131 is not currently on appeal or involved in an interference.

## STATUS OF CLAIMS

Claims 1-4, 6-7, 10-11, 15, 17-23, and 31 are the only pending claims.

Claims 5, 8-9, 12-14, 16, and 24-30 have been cancelled.

Claims 11, 15, and 18 are allowed.

Claim 7 is objected to.

Claims 1-4, 6, 10, 17, 19-23 and 31 are being appealed.

## STATUS OF AMENDMENTS

An Amendment under 37 C.F.R. § 1.116 was filed on December 22, 2004 in response to a Final Office Action dated September 22, 2004. In an advisory action mailed February 7, 2005, it was indicated that the proposed amendments submitted in the response dated December 22, 2004 were entered into the record.

A Supplemental Amendment under 37 C.F.R. § 1.116 is concurrently submitted herewith to rewrite dependent claim 7 in independent form. This amendment has not yet been entered into the record.

## SUMMARY OF CLAIMED SUBJECT MATTER

Human papillomaviruses (HPV) have been associated with a wide variety of biological phenotypes, from benign proliferative warts to malignant carcinomas[1]. HPV16 is a high-risk HPV type most frequently associated with *in situ* and invasive carcinomas of the cervix, vagina, vulva and anal canal. The present invention provides HPV16 genes that have been codon-optimized for expression in a human cellular environment, and their use with adenoviral vectors and/or plasmid vectors as vaccines.

The present invention provides HPV16 genes which were converted to nucleotide sequences having identical translated sequences but with alternative codon usage[2]. The present claims are directed to a synthetic polynucleotide comprising a nucleotide sequence encoding a codon-optimized HPV16 protein wherein said nucleotide sequence comprises codons that are optimized for expression in a human host, as recited in claim 1. Dependent claims 2-4, 6, 10, and 17 further define the scope of claim 1.

Independent claim 19 is directed to an adenoviral vaccine vector comprising a polynucleotide encoding a codon-optimized HPV16 protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein said polynucleotide is codon-optimized for expression in a human host cell. Dependent claim 20 further defines the scope of claim 19.

Independent claims 21 and 22 are directed to a shuttle plasmid vector and a vaccine plasmid, respectively, comprising a polynucleotide encoding a codon-optimized HPV16 protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein said polynucleotide is codon-optimized for expression in a human host cell. Claim 23 further defines the scope of claim 22.

Independent claim 31 is directed to a method of expressing a codon-optimized HPV16 gene in a recombinant host cell, by introducing a vector comprising a codon-optimized HPV16 polynucleotide into a suitable human host cell; and, culturing the host cell under conditions which allow expression of said HPV16 protein.

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[1] For review, *see* McMurray et al., *Int. J. Exp. Pathol.* 82(1): 15-33 (2001).

[2] Alternative codon usage, in the context of the present invention, is defined as set forth in Lathe, 1985 "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data: Theoretical and Practical Considerations" *J. Molec. Biol.* 183:1-12. The Lathe article defines optimal codons for high expression in human cells.

## GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The only issue to be reviewed on appeal is whether the specification enables a person skilled in the art to make and use the invention commensurate in scope with claims 1-4, 6, 10, 17, 19-23, and 31.

## Grouping of Claims

For reasons of this appeal, the claims are grouped according to the arguments set forth herein to overcome the grounds for rejection. The three groups of claims, each comprising claims that stand or fall together, are as follows:

Group I: Claims 1, 2, and 4

Group II: Claims 3, 6, 10, 17, and 19-23

Group III: Claim 31

## ARGUMENT

Summary of the Examiner's Position

The Appellants believe that the Examiner's reasons for holding that claims 1-4, 6-7, 10, 17, 19-23, and 31 are not enabled by the Specification can be summarized as follows: The Examiner alleges that the specification, "while being enabling for making and using a synthetic polynucleotide comprises (*sic*) SEQ ID Nos: 1, 2, 3, or 4, which are codons optimized for expression in human 293 cells, does not reasonably provide enablement for making and using a synthetic polynucleotide comprising unspecified codon that are optimized for expression in any cell of a human host."[3] The Examiner takes the position that the rejected claims are not enabled because codon optimization is not always successful at increasing expression of a particular gene and successful codon optimization of one gene is not predictive of the future success of a different gene.

Further the Examiner alleges that the Specification is additionally non-enabling for the scope of claim 31 because codon optimization of a gene in human cells is not predictive of successful codon optimization of that gene as expressed in host cells of other animals.

Summary of the Appellants' Position

The Appellants contend that the specification provides ample guidance to one of skill in the art on how to make and use the claimed invention without undue experimentation.

Detailed Discussion of the Appellants' Position

I. The Specification enables one skilled in the art to make and use the invention commensurate in scope with claims 1, 2, and 4.

Appellants submit that the Specification provides enablement for the full scope of claims 1-4, 6, 10, 17, 19-23, and 31 in accordance with 35 U.S.C. § 112, first paragraph.

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[3] *See* Office Action dated September 22, 2004, at page 3, paragraph 2.

The Examiner alleges that the Specification does not enable any person skilled in the art to make and use the invention commensurate in scope with the cited claims. Specifically, the Examiner states that the specification, "while being enabling for making and using a synthetic polynucleotide comprises (*sic*) SEQ ID Nos: 1, 2, 3, or 4, which are codons optimized for expression in human 293 cells, does not reasonably provide enablement for making and using a synthetic polynucleotide comprising unspecified codon that are optimized for expression in any cell of a human host."[4]

At the outset, Appellants note that the Office Action dated September 22, 2004, presented two arguments for non-enablement of the rejected claims, which can be summarized as follows: (1) codon optimization is not always successful at increasing expression of a particular gene and successful codon optimization of one gene is not predictive of the future success of a different gene and (2) successful optimization of a gene expressed in human 293 cells is not predictive of successful optimization of that gene in other human cells. Part (2) of this rejection, objecting to claimed subject matter encompassing expression of HPV16 proteins in cells other than human 293 cells, was modified in the Advisory Action dated February 7, 2005 to remove the scope limitation of human 293 cells.[5] As currently set forth, part (2) of the Examiner's argument alleges that the Specification fails to provide enablement for a process for expressing HPV16 proteins in a recombinant host cell of any animal, other than a human. As claims 1-4, 6, 10, 17, and 19-23 are product claims directed to a synthetic polynucleotide and not method claims, it is respectfully submitted that this aspect of the rejection is not relevant to these claims and, therefore, will not be discussed here.

The Supreme Court has interpreted the enablement requirement set forth in the first paragraph of 35 U.S.C. § 112 to require that, at the time of filing, one of skill in the art is able to practice the claimed invention without undue or unreasonable experimentation.[6] The Federal Circuit set forth the following factors to be considered in determining whether it would require undue experimentation to practice a claimed invention: (a) breadth of the claims; (b) nature of the invention; (c) the state of the prior art; (d) the level of one of ordinary skill; (e) the level of predictability in the art; (f) the amount of guidance provided by the inventor; (g) the existence of working examples; and (h) the quantity of experimentation needed to make or use

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[4] *See* Office Action dated September 22, 2004, at page 3, paragraph 2.

[5] *Infra* note 24 and Section III of the Argument

[6] *Mineral Separation v. Hyde* 242 U.S. 261, 270 (1916).

the invention based on the content of the disclosure.[7] The Court further opined that in establishing compliance with the enablement requirement, consideration of all of the evidence as a whole is required, and that basing a conclusion of nonenablement on a single factor alone is improper.[8]

The appealed claims are directed to synthetic polynucleotides that encode HPV16 proteins, the polynucleotides being codon-optimized for optimal expression in human cells. Appellants respectfully submit that the specification provides ample guidance to one of skill in the art on how to make and use the claimed invention. The specification exemplifies novel codon-optimized polynucleotides encoding HPV16 L1, E1, E2, and E7 proteins, which are particular embodiments within the scope of the claims. In that regard, particular attention is drawn to Examples 1-5 and Figures 1-5 and 9-12 of the Specification, which detail the construction and testing of numerous synthetic genes encoding the above HPV16 proteins.

Appellants further submit that the disclosure clearly teaches how one of skill in the art can obtain additional embodiments of the invention comprising codon-optimized HPV16 nucleotide sequences that are within the scope of the claims. See, for example, page 7, lines 9-28 of the Specification. Moreover, the Specification directs one of skill in the art to the disclosure of Lathe et al. (1985),[9] which describes the methodology needed to carry out codon optimization. Lathe describes in detail a statistical method for designing oligonucleotides with improved homology with a given target. The reference also describes the most frequent (or optimal in the cases of R and S) codon for each amino acid and the expected frequency of utilization within the human genome.[10] Using this data, it would be well within the abilities of those skilled in the art of molecular biology to design nucleotide sequences in which the codons have been optimized for human expression. Given the nature of molecular biology, the level of skill in the art is inherently high. Thus, the illustration of the invention with the given examples in the Specification, coupled with the prior art available at the time of filing, is more than adequate to teach one of skill in the art to make and use the claimed invention without undue experimentation.

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[7] *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (1988).
[8] 858 F.2d at 737, 8 USPQ2d at 1407.
[9] Lathe *et al.*, *J. Mol. Biol.* 183:1-12 (1985)
[10] Lathe *et al.*, at page 5, Col. 2, paragraph 3

That one of skill in the art could follow the teaching of the Specification to arrive at the claimed subject matter was conceded in the Office Action. In particular, the Examiner stated that "[t]he specification provides general teaching for means of obtaining such codons [optimized for expression in human cells] (e.g. Specification, page 7), and theoretically the claimed genus of codons could be made by the teaching of the specification."[11] The Examiner's recitation continues by arguing that although the claimed genus could be made by the teaching of the Specification, the claims are not enabled because the declaration of co-applicant William L. McClements,[12] submitted on June 10, 2004 (hereinafter "McClements declaration"), states that "codon-optimization is not always successful at increasing expression of a particular gene"[13] and that at the time of filing, applicants did not have a reasonable expectation of success, given the statements contained in the declaration.

Appellants respectfully submit that the Examiner's sole reliance on the McClements declaration to prove non-enablement is improper in light of the Federal Circuit's instruction in *In re Wands*[14], which counsels that all of the evidence must be considered as a whole. Appellants note that a rejection of then-pending claims 1-30 for lack of enablement was originally set forth in the Office Action dated June 5, 2002.[15] In a later Office Action, the Examiner indicated that the enablement rejection of all claims except now-cancelled claims 24-26 and 28 was rendered moot in view of the amendment dated July 24, 2003.[16] The current enablement rejection was not set forth until the Office Action dated September 22, 2004, in which the Examiner's argument in support of nonenablement is solely based on the McClements declaration, which was proffered in response to a rejection under 35 U.S.C. § 103.

It is important to note that the McClements declaration was submitted to show that the combination of prior art references cited in support of the §103 rejection did not provide one skilled in the art with the requisite "reasonable expectation of success" in selecting elements from each reference to piece together Applicants' invention at the time of filing. The declaration correctly points out that none of the cited references teaches codon optimization of HPV-16

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[11] *See* Office Action dated September 22, 2004, at page 4, lines 5-6.
[12] *See* Evidence Appendix.
[13] *See* Office Action dated September 22, 2004, at page 4, lines 6-11.
[14] 858 F.2d at 737, 8 USPQ2d at 1407.
[15] *See* Office Action dated June 5, 2002, at page5, last paragraph, to page 12, second paragraph.
[16] *See* Office Action dated September 9, 2003 at page 2, paragraph 3.

genes, and, therefore, it was not known that codon-optimization of HPV-16 genes would be successful prior to the present invention.[17]

The McClements declaration further summarizes art that was available to the skilled artisan at the time the instant application was filed, that identify inhibitory sequences and mRNA instability elements present in the HPV16 L1 and L2 genes, which could account for low expression levels of the HPV16 late genes. This art would lead one of skill in the art to believe that HPV 16 gene expression is limited by transcriptional regulation and mRNA instability, not codon usage, further supporting the notion that the "reasonable expectation of success" mandated by 35 U.S.C. § 103 was not present at the time of filing.

A complete reading of the McClements declaration and the accompanying response, submitted on June 10, 2004 demonstrates that the cited prior art, which discussed only codon-optimization of bovine papillomavirus type 1 (BPV-1) and green fluorescent protein (GFP) and not HPV16, was insufficient to render obvious the claimed invention. Though the McClements Declaration speaks to the state of the art at the time of filing the instant application, the Examiner relies on it to show that the teaching of the Specification is insufficient to enable one skilled in the art to make and use the present invention. Applicants respectfully submit that the enablement requirement of § 112 and the non-obviousness requirement of § 103 are separate and distinct requirements and the mere fact that there was no expectation of success based on the prior art does not mandate a conclusion of nonenablement, based on the teachings of the Specification.

Given the high level of skill in the art, it is respectfully submitted that the disclosure of the Specification is more than adequate to teach one of skill in the art to make and use the claimed invention without undue experimentation. Accordingly, Applicants respectfully request that the rejection of claims 1, 2, and 4 based upon the first paragraph of 35 U.S.C. § 112 be removed and the claims allowed.

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[17] *See* McClements Declaration at point 6, stating: "In each of these references, genes other than HPV-16 genes were codon-optimized. Both Frazer and Zolotukhin codon-optimized the gene encoding green fluorescent protein (GFP). Frazer additionally optimized only late genes from bovine papillomavirus 1 (BPV1). Based upon these references, it was not known that HPV-16 codon-optimization would be successful prior to the present invention, particularly given that GFP is not related at all to papillomavirus and that BPV-1 causes a different disease than HPV-16. More specifically, BPV-1 causes cutaneous fibropapillomas (plantar's warts), similar to HPV-1, wherein HPV16 causes infections of the genital mucosa."

II. The Specification enables one skilled in the art to make and use the invention commensurate in scope with claims 3, 6, 10, 17, and 19-23.

The Examiner alleges that the Specification does not enable any person skilled in the art to make and use the invention commensurate in scope with claims 3, 6, 10, 17, and 19-23 for reasons summarized in Section I of the Argument, *supra*. The appealed claims discussed in this Section are all limited in scope to synthetic polynucleotides encoding HPV16 L1, E1, E2, and E7 proteins.

As stated above, the Examiner has indicated that "theoretically the claimed genus of codons could be made by the teaching of the specification,"[18] but objects to the scope of the claims because "codon optimization is not always successful at increasing expression of a particular gene."[19] In response thereto, Appellants note that specific guidance and Examples are presented in the Specification which do, in fact, show successful codon-optimization of the HPV16 L1, E1, E2, and E7 genes. See, for example, Examples 2-5 and Figures 1-5 and 9-12 of the Specification.

Moreover, the Examiner failed to cite any objective evidence supporting the conclusion that codon optimization of these genes would be unsuccessful, contrary to what is actually shown in the application. Such evidence is mandated by *In re Marzocchi*,[20] in which the C.C.P.A. stated:

> A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement ... unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

The only argument set forth in the Office Action dated September 22, 2004 in support of non-enablement is that successful optimization of one gene is not predictive of successful

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[18] See Office Action dated September 22, 2004 at page 4, lines 4-6.
[19] See Office Action dated September 22, 2004 at page 4, lines 9-11.

optimization of a different gene. Applicants disclosure shows that the genes encompassed by the appealed claims were in fact successfully optimized, rendering this argument moot with respect to claims 3, 6, 10, 17, and 19-23.

It follows that one of skill in the art, based on the present disclosure, would be able to make and use codon-optimized HPV16 L1, E1, E2, and E7 genes in accordance with the limitation of the appealed claims. Consequently, in the absence of a well-reasoned statement, as required by *In re Marzocchi*[21], including a detailed explanation why the claimed invention allegedly lacks enablement, or why the truth or accuracy of the disclosure showing successful codon-optimization of the HPV16 L1, E1, E2, and E7 genes is doubted, the rejection must fail as a matter of law. As such, Applicants respectfully assert that a rejection under 35 U.S.C. § 112, paragraph one is improper. Accordingly, Applicants respectfully request that the rejection of claims 3, 6, 10, 17, and 19-23 be removed and the claims allowed.

III. The Specification enables one skilled in the art to make and use the invention commensurate in scope with claim 31.

As above, the Examiner alleges that the Specification does not provide enablement for claim 31 because (1) codon optimization is not always successful at increasing expression of a particular gene and successful codon optimization of one gene is not predictive of the future success of a different gene; and, additionally, because (2) codon optimization of a gene in human cells is not predictive of successful codon optimization of that gene as expressed in host cells of other animals.

With respect to point 1 of this rejection, Appellants respectfully submit that claim 31 is fully enabled by the Specification for reasons set forth in points I and II of the Argument, above.

With respect to point 2 of this rejection, Appellants initially note that this aspect of the rejection, which originally alleged that the Specification was enabling for codons

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[20] 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).
[21] *Id.*

optimized for expression in human 293 cells, but not in any cell of a human host[22], was modified to remove the scope limitation of human 293 cells[23]. As currently set forth in the Advisory Action dated February 7, 2005, it appears as though the Examiner is alleging that the Specification is enabling for human cells, but not for cells expressed in host cells of other animals.[24] It was further stated that this rejection is "particularly relevant to claim 31, wherein the claimed process encompasses a host cell of any animal."[25]

In response thereto, Appellants note that claim 31, which is directed to expression of a HPV16 protein in a recombinant host cell, clearly does not encompass expression of an HPV16 protein in a host cell of any animal. Contrary to the Examiner's position, it is evident that the scope of claim 31 is limited to expression in recombinant *human* cells by step (A) of the claimed process, which requires "introducing a vector comprising the synthetic polynucleotide of claim 1 into a suitable *human host cell*." (Emphasis added)[26]. Appellants further submit that because claim 31 is the only claim directed to expressing an HPV16 protein in a recombinant host cell, indeed, it is the sole pending method claim, this aspect of the rejection is not applicable to the remainder of the claims. As such, it is respectfully submitted that this aspect of the rejection is moot and respectfully request that the rejection be reversed and the claim allowed.

In view of the above discussion, the Appellants submit that it has been demonstrated that the full scope of claims 1-4, 6-7, 10, 17, 19-23, and 31 is enabled by Appellants Specification and respectfully request that the rejection of these claims based upon 35 U.S.C. § 112, paragraph 1, be removed and the claims allowed.

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[22] *See* Office Action dated September 22, 2004, at page 4, last sentence, stating "what is successful in 293 cells is not predictive of other human cell types."

[23] *See* Advisory Action dated February 7, 2005, paragraph 4.

[24] The Advisory Action states, at paragraph 4, that "upon reconsideration, this rejection has now been modified to remove the scope limitation of human 293 cells, thus claim now is only limited to any human cells, which is consistent with the teaching of the specification and applicants argument.

[25] *See* Advisory Action, lines 12-14.

[26] *See* Claims Appendix, claim 31. *See also,* Applicants Response dated December 22, 2004, at page 4, listing the currently pending text of claim 31.

## CONCLUSION

In view of the above, the Appellants respectfully request that the Board of Patent Appeals and Interferences reverse the outstanding rejection of claims 1-4, 6, 10, 17, 19-23 and 31 under 35 U.S.C. § 112, first paragraph.

Please charge deposit account 13-2755 for fees due in connection with this appeal brief. If any extensions of time are needed for the timely filing of the present appeal brief, appellants petition for such extensions and authorize the charging of deposit account 13-2755 for the appropriate fees.

Respectfully submitted,

By _____

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Reg. No. 48,878
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Date 2/22/2005

**CLAIMS APPENDIX**

1. A synthetic polynucleotide comprising a nucleotide sequence encoding a codon-optimized human papillomavirus serotype 16 (HPV16) protein wherein said nucleotide sequence comprises codons that are optimized for expression in a human host.

2. A polynucleotide according to Claim 1, wherein the protein is selected from the group consisting of: L1, L2, E1, E2, E4, E5, E6 and E7.

3. A polynucleotide according to Claim 2, wherein the protein is selected from the group consisting of: L1, E1, E2, and E7.

4. A polynucleotide according to Claim 2, wherein the polynucleotide is DNA.

6. A polynucleotide according to Claim 4, wherein the protein is an HPV16 L1 protein.

10. A polynucleotide according to Claim 4, wherein the polynucleotide encodes an HPV16 E1 protein.

17. A polynucleotide according to Claim 4, wherein the protein is an HPV16 E7 protein.

19. An adenoviral vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising:

A) a polynucleotide encoding a codon-optimized HPV16 protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein said polynucleotide is codon-optimized for expression in a human host cell; and

B) a promoter operably linked to the polynucleotide.

20. A vector according to Claim 19, wherein the adenoviral genome also contains a deleted E3 region.

21. A shuttle plasmid vector comprising a plasmid portion and an adenoviral portion, the adenoviral portion comprising: an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising:

A) a polynucleotide encoding a codon-optimized HPV16 protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein said polynucleotide is codon-optimized for expression in a human host cell; and

B) a promoter operably linked to the polynucleotide.

22. A vaccine plasmid comprising a plasmid portion and an expression cassette portion, wherein the expression cassette portion comprises:

A) a polynucleotide encoding a codon-optimized HPV16 protein selected from the group consisting of L1, E1, E2, and E7 proteins, wherein said polynucleotide is codon-optimized for expression in a human host cell; and

B) a promoter operably linked to the polynucleotide.

23. A plasmid according to Claim 22, wherein the plasmid portion is V1Jns.

31. A process for expressing an HPV16 protein in a recombinant host cell, comprising:

(A) introducing a vector comprising the synthetic polynucleotide of claim 1 into a suitable human host cell; and,

(B) culturing the host cell under conditions which allow expression of said HPV16 protein.

## EVIDENCE APPENDIX

A Declaration of co-inventor William L. McClements under 37 C.F.R. § 1.132 (supported by Exhibits A-G) was concurrently submitted with an Amendment under 37 C.F.R. § 1.111 on June 10, 2004. A Final Office Action, mailed on September 22, 2004, indicates that the Declaration and supporting documentation (Exhibits A-G) were entered into the record.

1 DOCKET
2 FILE

CERTIFICATE OF MAILING

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail, in an envelope addressed to the Commissioner For Patents, P.O. Box 1450, Alexandria, VA 22313-1450

Nancy E. York — Signature — June 10, 2004

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicants: | Neeper, M. P. et al. | |
| Serial No.: | 09/642,405 - Case No.: 20413Y | Art Unit: 1632 |
| Filed: | August 21, 2000 | Examiner: Li, Q. J. |
| For: | SYNTHETIC HUMAN PAPILLOMAVIRUS GENES | |

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JUN 11 2004
LUATSCHEPISI

Commissioner for Patents
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DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, William L. McClements, Hereby declare that:

1. I am a named as co-applicant of U.S. Application Serial Number 09/642,405, filed August 21, 2000, which claims priority from U.S. Provisional Patent Application No. 60/150,728, filed August 25, 1999, and also claims priority from U.S. Provisional Patent Application No. 60/210,143, filed June 7, 2000.

2. I am currently a Senior Investigator at Merck & Co., Inc., and have been since October 2002. Additional research positions that I held at Merck consist of the positions of Senior Research Fellow from July 1995 to October 2002, Research Fellow from May 1988 to July 1994, and Senior Research Virologist from 1983 to April 1988. Prior to working at Merck, I was employed as a Senior Scientist by Genex Corporation in Gaithersburg, Maryland. My primary responsibilities during my employment at Merck include research and development in the field of microbial vaccines. During my scientific career, I have co-authored over thirty-five

peer-reviewed publications in the fields of virology and molecular and cellular biology (see Exhibit A). I have also presented my research at numerous scientific meetings (see Exhibit B). Additionally, I am an inventor of subject matter claimed in 6 patent families and hold a number of issued patents in the United States and other jurisdictions (See Exhibit C).

3. I have reviewed the present Application, the pending claims, and the Office Action mailed February 10, 2004 (hereinafter "Office Action"). The pending claims are drawn to synthetic polynucleotides comprising a nucleotide sequence encoding a codon-optimized human papillomavirus serotype 16 (HPV16) protein, adenoviral vaccine vectors comprising an adenoviral genome comprising a polynucleotide encoding a codon-optimized HPV16 protein, shuttle plasmid vectors comprising a nucleotide sequence encoding a codon-optimized HPV16 protein, a vaccine plasmid comprising a nucleotide sequence encoding a codon-optimized HPV16 protein, and a process for expressing an HPV16 protein in a recombinant host cell.

4. I understand that the Examiner has rejected pending claims 1-4, 6, 10, 17, 21, 22, and 30, *inter alia*, on the grounds that it would have been obvious to one of skill in the art at the time the invention was made to use the methods of Zolotukhin et al. (U.S. Patent No. 5,874,304; hereinafter, "Zolotukhin"), Frazer et al. (U.S. Patent No. 6,489,141, hereinafter "Frazer") or WO 99/02694 (hereinafter "Frazer PCT") in view of Ludmerer et al., (U.S. Patent No. 5,952,216; hereinafter "Ludmerer") and Apt et al. (U.S. Patent No. 6,399,383, hereinafter "Apt") "for efficiently producing HPV-16 proteins with a reasonable expectation of success." (Office Action, page 6, lines 7-10). The Examiner further alleges that "[t]he ordinary skilled artisan would have been motivated to modify the claimed invention because the improved efficiency of protein production." (Office Action at page 6, lines 10-11). I respectfully disagree.

5. It is my contention that it would not have been obvious to combine elements from Zolotukhin, and Frazer in view of Ludmerer and Apt to arrive at the instant invention because, at the time the application was filed, we did not have an "expectation of success" based on our previous experience with codon-optimization. Prior to filing the instant application, I participated in the codon-optimization of the gD and gB genes from Herpes simplex virus type 2 (HSV2) for high level expression in human cells. Analysis of expression of the optimized HSV2 genes compared to their natural counterparts indicated that gene expression did not improve upon codon-optimization, but rather stayed the same. Prior to the time of filing, I was also aware of my colleagues' attempts to improve expression of genes from tuberculosis

(TB). In that case, expression of the "optimized" versions of the TB genes was actually lower as compared to the wild-type equivalents.

6. It is my contention that, as stated above, codon-optimization is not always successful at producing increased gene expression in a cell type of interest and that successful codon-optimization of one virus is not predictive of future success with a different virus. In the present case, the Examiner has cited Frazer and Zolotukhin in view of Ludmerer and Apt in support of the assertion that it would have been obvious to codon-optimize genes from HPV-16. In each of these references, genes other than HPV-16 genes were codon-optimized. Both Frazer and Zolotukhin codon-optimized the gene encoding green fluorescent protein (GFP). Frazer additionally optimized only late genes from bovine papillomavirus 1 (BPV1). Based upon these references, it was not known that HPV-16 codon-optimization would be successful prior to the present invention, particularly given that GFP is not related at all to papillomavirus and that BPV-1 causes a different disease than HPV-16. More specifically, BPV-1 causes cutaneous fibropapillomas (plantar's warts), similar to HPV-1, wherein HPV16 causes infections of the genital mucosa.

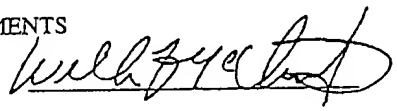
7. It is my contention that art existed at the time of filing the present application that would lead one of skill in the art to believe that HPV 16 gene expression is limited by transcriptional regulation and mRNA instability, not codon usage. For example, Sokolowski and colleagues (*J. Virol.* 72(2): 1504-1515 (1998); Exhibit D) identified two *cis*-acting inhibitory sequences in the HPV16 L2 gene, which led to reduced cytoplasmic mRNA stability. In contrast, Sokolowski *et al.* also showed that such inhibitory sequences were not found in HPV1 L1 or L2 genes, which are similar to the BPV-1 virus disclosed by Frazer, discussed above. Another study by Kennedy *et al.* (*J. Virol.* 64(4): 1825-1829 (1990), Exhibit E) also disclosed the presence of a negative regulatory element located upstream of the HPV type 16 late mRNA polyadenylation signals, which they concluded "probably acts as a mRNA instability sequence." *See* Kennedy at page 1828, last paragraph. Tan *et al.* (*J. Virol.* 69(9): 5607-5620 (1995), Exhibit F) also reported the presence of an inhibitory element in the open reading frame of HPV16 L1. Additional discussion on the tightly regulated expression of papillomavirus late genes and the affect of *cis*-acting negative RNA elements on papillomavirus late gene expression was reviewed by Stefan Schwartz in 1998 (*Seminars in Virology* 8: 291-300, Exhibit G).

8. It is my understanding that the Examiner has also rejected pending claims 19, 20, and 23, on the grounds that it would have been obvious to one of skill in the art at the time the invention was made to use the methods of Zolotukhin and Frazer in view of Ludmerer and Apt, and further in view of Ertl et al. (U.S. Patent No. 6,019,978, hereinafter "Ertl") and Donnelly et al. (*J. Infect. Diseases* 713: 314-20 (1996); hereinafter "Donnelly"). The rejected claims are drawn to adenoviral vaccine vectors comprising an expression cassette comprising a polynucleotide encoding a codon-optimized HPV16 protein, wherein said polynucleotide is codon-optimized for expression in a human host cell, and a vaccine plasmid comprising an expression cassette comprising a polynucleotide encoding a codon-optimized HPV16 protein, wherein said polynucleotide is codon-optimized for expression in a human host cell. I respectfully disagree with the Examiner's assertion for reasons appearing in paragraphs 5-7 above.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this application for patent or any patent issuing thereon.

Furthermore, Declarant sayeth not.

Declarant's full name: WILLIAM LYTLE MCCLEMENTS



Date: 10 June 2004

attachments: Exhibits A-G

**EXHIBIT A**

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**EXHIBIT C**

Patents and Patent Applications

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# mRNA Instability Elements in the Human Papillomavirus Type 16 L2 Coding Region

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**Human papillomavirus capsid proteins L1 and L2 are detected only in terminally differentiated cells, indicating that expression of the L1 and L2 genes is blocked in dividing cells. The results presented here establish that the human papillomavirus type 16 L2 coding region contains *cis*-acting inhibitory sequences. When placed downstream of a reporter gene, the human papillomavirus type 16 L2 sequence reduced both mRNA and protein levels in an orientation-dependent manner. Deletion analysis revealed that the L2 sequence contains two *cis*-acting inhibitory RNA regions. We identified an inhibitory region in the 5′-most 845 nucleotides of L2 that acted by reducing cytoplasmic mRNA stability and a second, weaker inhibitory region in the 3′ end of L2. In contrast, human papillomavirus type 1 L1 and L2 genes did not encode strong inhibitory sequences. This result is consistent with observations of high virus production in human papillomavirus type 1-infected tissue, whereas only low levels of human papillomavirus type 16 virions are detectable in infected epithelium. The presence of inhibitory sequences in the L1 and L2 mRNAs may aid the virus in avoiding the host immunosurveillance and in establishing persistent infections.**

Human papillomaviruses (HPVs) are nonenveloped DNA tumor viruses that can induce a variety of proliferative lesions upon infection of epithelial cells (28, 53, 70, 72). To date, more than 70 different HPV types have been identified (24). Each of these types infects either mucosal or cutaneous epithelium at distinct anatomical sites. Members of a subset of the HPV types are etiological agents of cancers, e.g., HPV-16, and are referred to as high-risk types, whereas certain HPV types are rarely or never found in cancers, e.g., HPV-1, and are referred to as low-risk types (28, 35, 53, 71). HPV-1 infects cutaneous epithelium at the plantar surface of the foot, whereas HPV-16 shows tropism for mucosal epithelial cells.

The production of HPV virions is strictly linked to the differentiation stage of the infected epithelial cell, and viral late-gene products, L1 and L2 (Fig. 1), are detected primarily in the terminally differentiated cells in the upper layers of the epithelium (11, 23, 32, 33, 53, 56, 61). One reason for the restriction of HPV late-gene expression to terminally differentiated cells and for the differences observed in the levels of expression of late-gene products from various HPV types may be the presence of negative regulatory elements in the HPV late mRNAs. Such elements were originally described by Kennedy et al. (29, 30), who reported the identification of an inhibitory sequence in the HPV-16 late 3′ untranslated region (UTR) (Fig. 1) which acted by reducing mRNA stability in vitro. Other investigators proposed that the activity of this negative regulatory element required the presence of a 5′ splice site-like sequence (21). In an attempt to produce HPV-16 L1 from eucaryotic expression plasmids encoding L1 cDNAs, we reasoned that deletion of the negative sequence in the late 3′ UTR would allow high L1 production. However, deletion of the late 3′ UTR from an HPV-16 L1 cDNA did not result in the production of detectable levels of L1 (58), indicating that the L1 coding region itself contained sequences that inhibit L1 production. These sequences acted in *cis* and inhibited the expression of a reporter gene to the extent of several hundred-fold (58). Subsequent experiments demonstrated that inhibitory sequences were located primarily in the 5′ half of the L1 coding region and spanned several hundred nucleotides (58).

In the work described here, we investigated if HPV-16 L2 (Fig. 1) contains sequences that negatively affect L2 expression levels. The results presented here demonstrate that the HPV-16 L2 coding region contains *cis*-acting inhibitory RNA sequences that act by reducing mRNA and protein levels. A sequence in the 5′ end of HPV-16 L2 acted as an mRNA instability determinant, and a weaker, posttranscriptionally active sequence was found in the 3′ end of L2. We also show that the HPV-1 L1 and L2 coding regions do not contain strong inhibitory sequences. HPV-1 virions are easily detected in vivo, whereas HPV-16 virions are not. Therefore, the presence of inhibitory sequences in L1 and L2 correlates with the amounts of virus produced in vivo.

## MATERIALS AND METHODS

Plasmid constructions. The following plasmids have been described elsewhere: pE55 (59), pNLCATW (58), and pCS1X (58); pC16L1(A) and pC16L1(S) have been described as pCATL1A and pCATL1S (58), respectively, and pT7-16L2 has been described as pT7L2 (25).

pH16L2 was generated by excising a *Bst*HII-*Kpn*I fragment from pT7-16L2, followed by insertion into *Bst*HII- and *Kpn*I-digested pNLCATW (58), thereby replacing the chloramphenicol acetyltransferase (CAT) gene with the HPV-16 L2 open reading frame (ORF). To generate pCMV16L2, the HPV-16 L2 coding region was PCR amplified with oligonucleotides L2START (5′-CAGCGCGCC CTTAACAATGCGACACAAACG-3′) and L2STOP (5′-CAGTCGACCGTG GCCTCACTAGGCAGCC-3′) and inserted into *Hpa*I-digested, calf intestinal alkaline phosphatase (CIAP)-treated pLNCX (36). pC16L2(S) and pC16L2(A) were produced by subcloning, in the sense and antisense orientations, respectively, an HPV-16 L2 DNA fragment that had been PCR amplified from pT7-16L2 with oligonucleotides L2START and L2STOP into *Asp*718-digested, Klenow fragment-treated pNLCATW (58). pC16L2-Stop was constructed by annealing oligonucleotide GC-BAMHI (5′-CGCGCGGGGGGGGGGGGG GGGGGGGGGATCCCCCCCCCCCCCCCCCCCCCCCG-3′), followed by subcloning into pC16L2(S) that had been digested with *Bst*HII and treated with CIAP. Digestion of pC16L2(S) with *Mlu*I and *Bst*HII, followed by T4 DNA polymerase treatment and religation, generated p16L2ΔC. pCL2D was gener-

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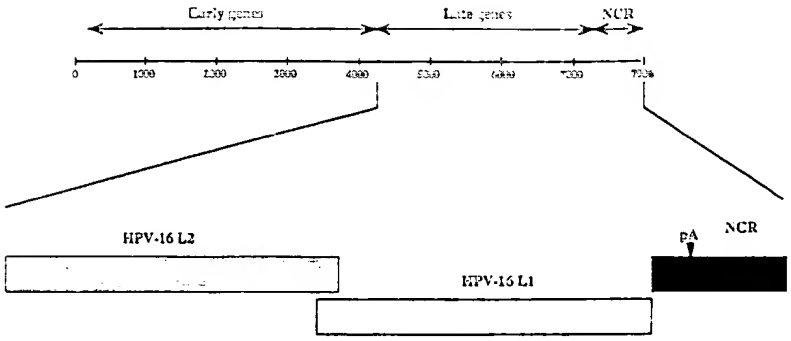


FIG. 1. Genomic map of HPV-16. Numbers indicate nucleotide positions (51), the shaded box indicates the L2 coding region, and the white box indicates the L1 coding region. NCR, noncoding region, containing the late 3' UTR; pA, HPV-16 late polyadenylation signal.

ated by digestion of pC16L2(S) with *Sal*I and *Spe*I and religation. To generate pCL2C, pC16L2(S) was digested completely with *Sal*I and partially with *Spe*I and religated. A fragment that had been PCR amplified from pT7-16L2 with oligonucleotides L2START and L2M (5'-CGTCGACCTGGAGCTATATTAATAC-3') was ligated into pBluescript that had been digested with *Eco*RV and treated with CIAP, generating pKSL2B. To generate pCL2B, a *Bss*HII-*Sal*I fragment from pKSL2B was ligated to pC16L2(S) that had been digested with *Mlu*I and *Sal*I. pCL2A was constructed by digestion of pC16L2(S) with *Spe*I, followed by religation. pCL2R1, pCL2R2, pCL2R3, pCL2R5, and pCL2R7 were constructed by insertion into *Asp*718-digested, Klenow fragment-treated pNLCATW (58) of HPV-16 L2 DNA fragments that had been PCR amplified with the following primer pairs: L2D (5'-CGTCGACGGAATTAATGAAGGAGCTTGG-3') and L2B (5'-CACGCGTCAGTAACTAGTAGCACACCCA-3'), L2C (5'-CACGC GTAATATAGCTCCAGATCCTGAC-3') and L2STOP, L2B and L2G (5'-CG TCGACGGATCAATAGTACTTAAA-3'), L2E (5'-CACGCGTCTATTGATC CTGCAGAAG-3') and L2STOP, and L2C and L2D. To generate pL2HU(S) and pL2HU(A), HPV-16 L2 sequences were PCR amplified with oligonucleotides L2B and L2STOP and inserted in the sense and antisense orientations, respectively, into *Stu*I-digested pNLCATW (58).

pC1L1(S) and pC1L1(A) were generated by subcloning into *Asp*718-digested, Klenow fragment-treated, CIAP-treated pNLCATW (58), in the sense and antisense orientations, respectively, HPV-1 L1 sequences that had been PCR amplified from pHPV-1 (17) with oligonucleotides H1L1STOP (5'-GTTATATAG AATTCATACTAAGCC-3') and H1L1START (5'-AGCGTCGACAAAGAGC TTATGT-3'). pC1L2(S) and pC1L2(A) were generated by subcloning into *Asp*718-digested, Klenow fragment-treated pNLCATW (58), in the sense and antisense orientations, respectively, HPV-1 L2 sequences that had been PCR amplified with H1L2S (5'-CGTCGACGTAACAAATGTATCGCCTACG-3') and H1L2A (5'-AGAATTCCATTATACATAAGCTCTTTTACG-3').

pHCMVtat was constructed by subcloning a *Sal*I-*Hpa*I human immunodeficiency virus type 1 (HIV-1) Tat-encoding fragment from pNL147 (48) into *Sal*I- and *Hpa*I-digested pCH16pA (58). pKSNLCAT was generated by ligation of a *Hin*dIII-*Eco*RI fragment from pNLCATW into pBluescript (Stratagene) that had been digested with *Hin*dIII and *Eco*RI.

**Cells, transfections, and CAT ELISA.** For transfection of adherent cells (HLtat [48], 293, NIH 3T3, BHK-21, and CV-1), $3 \times 10^5$ cells were seeded per 60-mm-diameter plate 24 h prior to transfection. Plasmid pHCMVtat, producing HIV-1 Tat, was included in transfections. Transient transfections were carried out by the calcium phosphate coprecipitation technique (22) as described previously (60). For suspended cells (Jurkat and U937), $10^6$ cells were transfected with Lipofectamine (Life Technologies) according to the manufacturer's instructions. The cells were harvested 20 to 48 h posttransfection, and the amount of CAT protein was quantified in a CAT antigen capture enzyme-linked immunosorbent assay (ELISA; Boehringer GmbH). pCS1X (58) was included as an internal control in each transfection experiment, and secreted alkaline phosphatase (SEAP) activity was determined as previously described (58). In transfections executed for downstream RNA analysis, pHCMVtat was included as an internal control. When the vaccinia virus T7 RNA polymerase expression system (20) was used, cells were infected with $0.5 \times 10^6$ PFU of recombinant vaccinia virus vTF7-3 (20) expressing T7 RNA polymerase 1 to 2 h prior to transfection. Each experiment was repeated at least three times, and mean values of representative results are shown.

**RNA preparation and primer extension.** Total cytoplasmic RNA was prepared from transfected HeLa or HLtat cells as previously described (60). For fractionation and preparation of nuclear RNA, the cells were lysed directly in culture dishes with Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 0.65% [NP-40 Sigma]). The nuclei were washed three times in NP-40 lysis buffer and then scraped off the culture dishes into 250 μl of NP-40 lysis buffer with a rubber policeman. Nuclei from two 60-mm-diameter culture dishes were pooled. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.2%, and nuclei were lysed on ice for 5 min, with repeated vortexing. Following freezing-thawing on dry ice and at 37°C, the samples were treated with DNase I for 10 min at 37°C. The samples were extracted twice with an equal volume of phenol-chloroform [at a ratio of 1:1; phenol was equilibrated in 1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 6.0)], followed by extraction with chloroform and ethanol precipitation. Pellets were resuspended in water, RNA was quantified by spectrophotometry, and the quality of the RNA was checked on agarose gels.

Primer extension was carried out by coprecipitating 50 μg of nuclear or cytoplasmic RNA with $10^5$ cpm of [$\gamma$-$^{32}$P]ATP-labelled oligonucleotides NL-PX2 (5'-GGGCACACACTACTTTGAG-3'), CMV-PX3 (5'-TGGATCGGTCCCG GTGTCTT-3'), and 47S-PX6 (5'-GCCAGAGCCCCGCGCGCATC-3'). Oligonucleotide NL-PX2 hybridizes to the 5' end of mRNA transcribed from the HIV-1 long terminal repeat (LTR) promoter, oligonucleotide CMV-PX3 hybridizes to the 5' end of mRNA transcribed from the human cytomegalovirus (CMV) immediate-early promoter, and oligonucleotide 47S-PX6 hybridizes to the 5' end of the 47S rRNA precursor transcript. Annealing was performed by resuspending the RNA pellet in 8 μl of 1× RT buffer (50 mM Tris-Cl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate [dNTP]), followed by incubation at 65°C for 1 min, at 37°C for 1 min, and on ice for 1 min. cDNA synthesis was carried out by adding 8 μl of 1× RT mixture (50 mM Tris-Cl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each dNTP, 5 U of Molony murine leukemia virus reverse transcriptase [Life Technologies] per μl, 0.25 U of RNA Guard [Pharmacia] per μl), followed by incubation at 42°C for 60 min. The reaction was stopped by RNase treatment (1 μg of RNase A and 20 U of RNase $T_1$ [Ambion]) at 37°C for 10 min, followed by ethanol precipitation. The pellet was resuspended in formamide loading dye (80% deionized formamide, 1 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol), and the suspension was boiled for 2 min. The reaction products were separated on 6% polyacrylamide–urea gels. Gels were analyzed by autoradiography, and RNA levels were quantified with a PhosphorImager (Molecular Dynamics). Each experiment was repeated at least three times, and representative results are shown.

**Northern RNA blotting.** Northern RNA blotting was performed essentially as described previously (58). An antisense [$\alpha$-$^{32}$P]UTP-labelled riboprobe was generated from *Hin*dIII-linearized pKSNLCAT as described previously (68). The synthetic RNA was complementary to the 5' ends of mRNAs produced from pNLCATW-derived plasmids and contained 180 nucleotides (nt) of the HIV-1 5' LTR and 213 nt of the 5' end of the CAT gene.

**Extraction of poly(A)$^+$ mRNA and reverse transcription (RT)-PCR.** Cytoplasmic poly(A)$^+$ mRNA was isolated with Dynabeads Oligo $(dT)_{25}$ (Dynal A. S.) as described previously (60). Briefly, transfected cells were lysed for 5 min on ice in NP-40 lysis buffer. Cytoplasmic and nuclear fractions were separated by centrifugation at 8,000 × *g* for 2 min. Supernatants were incubated with an equal volume of 2× binding buffer (20 mM Tris-HCl [pH 7.5], 1.0 M LiCl, 2 mM EDTA, 0.5% SDS) containing 400 μg of Dynabeads Oligo $(dT)_{25}$. After three washes in washing buffer (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 2 mM EDTA), poly(A)$^+$ mRNAs were eluted from the beads with elution buffer (2 mM EDTA [pH 7.5]) at 65°C for 2 to 3 min and stored at −70°C until use.

RT-PCR was performed as previously described (60). Briefly, fourfold serially diluted cytoplasmic poly(A)$^+$ mRNA was reverse transcribed at 42°C for 1 h in a total reaction volume of 30 μl with random hexamers. Five microliters of the cDNA product was PCR amplified in a 100-μl reaction volume with oligonucle-

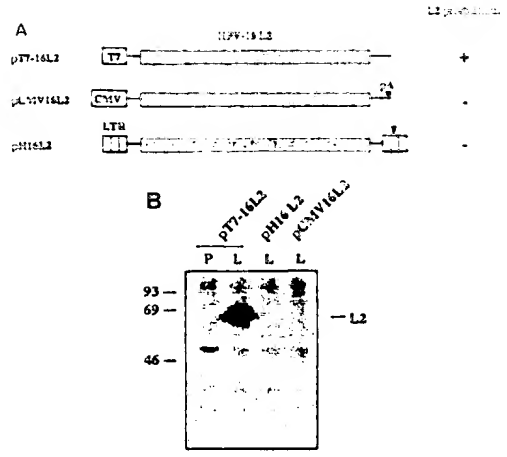


FIG. 2. (A) Structures of the L2 expression plasmids. Shaded boxes indicate the HPV-16 L2 coding region, striped boxes represent HIV-1 LTRs, and triangles represent poly(A) signals (pA). Plasmid names are indicated on the left. T7, bacteriophage T7 RNA polymerase promoter; CMV, CMV immediate-early promoter. (B) Radioimmunoprecipitation of HPV-16 L2 from HeLa cells infected with recombinant vaccinia virus vTF7-3 (20) and transfected with pT7-16L2 or HLtat cells (48) transfected with pH16L2 or pCMV16L2. P, preimmune guinea pig serum; L, guinea pig anti–HPV-16 L2 peptide antiserum (18). Numbers indicate molecular masses in kilodaltons.

otides CATS-2 (5′-CGTCTCAGCCAATCCCTGGGTG-3′) and CATA (5′-CT ATTAGGCCCCGCCCTGCCACTC-3′) to detect cDNA of the CAT or CAT-HPV-16 L2 hybrid mRNA or EP (5′-AGGTGACGGTACAAGGGTCTCAGA AA-3′) and EW (5′-CCCACCATGTTCTTTCAAAGGC-3′) to detect cDNA of the equine infectious anemia virus (EIAV) *gag* mRNA produced from the internal control plasmid pE55 (59). PCR was performed in a total reaction volume of 100 μl for 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. A 10-μl sample from each RT-PCR was analyzed by electrophoresis on 5% polyacrylamide gels.

Radioimmunoprecipitation. Transfected cells were starved for 30 min in Met-free medium containing 0.5% fetal calf serum, followed by metabolic labelling for 1 h with 200 μCi of [$^{35}$S]Met. The cells were washed and lysed in ice-cold RIPA buffer (25 mM Tris-HCl [pH 7.4], 75 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS). After three freezing-thawings, the cell extracts were centrifuged, and supernatants were collected, mixed with normal guinea pig serum, and incubated for 30 min at 4°C. Protein A-Sepharose (Pharmacia) beads were added, and incubation was continued for 1 h, followed by centrifugation and collection of supernatants. Normal guinea pig serum or guinea pig anti–HPV-16 L2 peptide antiserum (18) was added, and incubation was performed at 4°C for 16 h, followed by the addition of protein A-Sepharose beads and continued incubation for 3 h. The samples were heated, loaded onto 10% polyacrylamide–SDS gels (acrylamide/bisacrylamide ratio, 29:1) under reducing conditions, and electrophoresed at 180 V. The gels were dried and autoradiographed at −70°C.

## RESULTS

**The HPV-16 L2 protein can be efficiently produced in HeLa cells by use of the vaccinia virus T7 RNA polymerase-based expression system but not by use of eucaryotic expression plasmids.** We first attempted to express HPV-16 L2 (Fig. 1) from plasmids containing the HIV-1 LTR promoter (pH16L2) (Fig. 2A) or the CMV promoter (pCMV16L2) (Fig. 2A), which we have used previously for high expression of other virus genes, e.g., those encoding EIAV and HIV-1 proteins (48, 49, 59). However, the levels of L2 produced from these plasmids were undetectable (Fig. 2). In contrast, transfection of plasmid pT7-16L2, which contains the bacteriophage T7 promoter (Fig. 2A), into HeLa cells infected with a recombinant vaccinia virus producing T7 RNA polymerase (20) yielded high levels of L2 protein (Fig. 2). In the latter case, transcription of the plasmid occurs in the cytoplasm, while in the former case, nuclear factors are required. We do not know if the high L2 expression levels observed in the vaccinia virus T7 RNA polymerase expression system are a result of the bypassing of the nucleus, overall high transcription levels in this expression system, or interactions between vaccinia virus and the infected cell. We obtained similar results previously using the HPV-16 L1 gene (58). Our results indicated that the HPV-16 L2 coding region contains inhibitory sequences.

The HPV-16 L2 coding region contains *cis*-acting inhibitory sequences that act in an orientation-dependent manner. To investigate if the HPV-16 L2 coding region contains sequences that inhibit gene expression, the entire HPV-16 L2 coding region was inserted, in sense and antisense orientations, downstream of the CAT reporter gene in plasmid pNLCATW (58), resulting in plasmids pC16L2(S) and pC16L2(A), respectively (Fig. 3). These plasmids were separately transfected in triplicate into HLtat cells (48) in the presence of the SEAP-producing plasmid pCS1X (58), included as an internal control for transfection efficiency. The standard deviation was less than 30% in all experiments shown, and all plasmids were analyzed in a minimum of three independent transfection experiments. Mean values of CAT levels produced after triplicate transfections revealed that pC16L2(S) produced 49-fold-lower levels of CAT than pNLCATW (58), whereas pC16L2(A) and

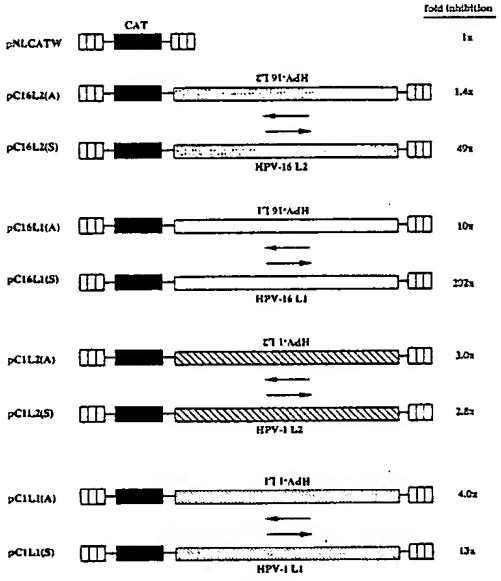


FIG. 3. Schematic structures of CAT expression plasmids. The HIV-1 LTR (striped boxes), the CAT gene, and the HPV-1 or HPV-16 L1 and L2 sequences are indicated. Plasmids contained the L1 or L2 ORF from the first ATG to the translational stop codon. Plasmid names are shown on the left. Arrows indicate sense (→) and antisense (←) orientations. The depicted plasmids were transfected in triplicate into HLtat cells, and CAT levels were monitored in a CAT ELISA and normalized to SEAP levels produced from the internal control plasmid pCS1X (58). The mean CAT values obtained after triplicate transfections with pNLCATW (58) were divided by the mean CAT values obtained after triplicate transfections with the indicated plasmids to yield fold inhibition.

TABLE 1. The negative element in HPV-16 L2 is active in cells of different origins

| Cell line | Origin | Fold inhibition |
|---|---|---|
| HeLa | Human epithelioid | 126 |
| 293 | Human epithelial morphology | 14 |
| Jurkat | Human T cell | 59 |
| U937 | Human monocyte | 14 |
| CV-1 | Monkey fibroblast morphology | 39 |
| BHK-21 | Hamster fibroblast morphology | 55 |
| NIH 3T3 | Mouse fibroblast | 68 |

pNLCATW (58) produced similar levels of CAT (Fig. 3). These results demonstrated that the HPV-16 L2 coding region contains *cis*-acting inhibitory sequences that acted in an orientation-dependent manner to reduce CAT production. For comparison, we analyzed the effect on CAT expression of the HPV-16 L1 coding region. The results demonstrated that pC16L1(S) produced 202-fold-lower CAT levels than pNLCATW (58) and that the presence of L1 in an antisense orientation had a lower inhibitory effect (Fig. 3), as we described previously (58). The HPV-16 L1 sequence had stronger inhibitory activity than the L2 sequence.

**The HPV-1 L1 and L2 coding regions do not contain strong inhibitory sequences.** We next investigated if the presence of inhibitory sequences in the L1 and L2 coding regions is a general property of HPVs. The L1 and L2 coding sequences from HPV-1 were separately inserted in the sense and antisense orientations downstream of the CAT gene in pNLCATW (58), resulting in pC1L1(S), pC1L1(A), pC1L2(S), and pC1L2(A) (Fig. 3). These plasmids were transfected into HLtat cells (48), and the levels of CAT were determined. The results showed that HPV-1 L1 and L2 inhibited CAT expression 13- and 3-fold, respectively (Fig. 3), demonstrating that the HPV-1 L1 and L2 coding sequences encode only low inhibitory activity. The ratios between CAT levels produced from each plasmid containing HPV sequences in the sense orientation and CAT levels produced from the corresponding plasmid containing HPV sequences in the antisense orientation were 0.32 and 1.1 for the HPV-1 L1 [pC1L1(S)/pC1L1(A)] and L2 [pC1L2(S)/pC1L2(A)] plasmids, respectively, whereas the corresponding ratios for the HPV-16 L1 [pC16L1(S)/pC16L1(A)] and L2 [pC16L2(S)/pC16L2(A)] plasmids were 0.05 and 0.03, respectively. We concluded that the HPV-16 L1 and L2 coding regions contain strong inhibitory sequences located on the coding strand; the HPV-1 L2 sequence appeared to lack significant inhibitory activity, whereas the HPV-1 L1 sequence had weak inhibitory activity.

**The inhibitory sequences in HPV-16 L2 are active in cells of different origins.** We transfected pC16L2(A) or pC16L2(S) (Fig. 2A) into cell lines of different origins (Table 1) to test if the inhibitory activity of the L2 sequences was restricted to epithelial cells. Table 1 shows a 14- to 126-fold difference in CAT production between pC16L2(A) and pC16L2(S) in the cell lines, indicating that the inhibitory sequences were functional in different cell types and in cells from different species. However, the inhibitory element in HPV-16 L2 acted most efficiently in human epithelial cells. The results indicated that the regulatory mechanism involving the HPV-16 L2 sequences is evolutionarily conserved and is not cell type specific.

**The HPV-16 L2 coding region contains *cis*-acting sequences that reduce cytoplasmic and nuclear mRNA levels.** To further investigate the inhibitory effect of the HPV-16 L2 coding sequence, plasmids pC16L2(S), pC16L2(A), and pNLCATW were separately transfected into HLtat cells in the presence of the internal control plasmid pHCMVtat (see Materials and Methods), and cytoplasmic mRNA levels were monitored by primer extension. The results revealed that pNLCATW and pC16L2(A) produced similar mRNA and protein levels, whereas pC16L2(S) produced significantly lower mRNA and protein levels than pC16L2(A) and pNLCATW (Fig. 4A), respectively. The differences were 20- to 13-fold at the mRNA level and 133- to 147-fold at the protein level. Quantitation of mRNA and protein levels in cells transfected with serially diluted pNLCATW verified that the analysis was performed in the linear range of the assays (Fig. 4B). In addition, we analyzed cytoplasmic poly(A)$^+$ mRNA by RT-PCR with fourfold serially diluted mRNA. The results demonstrated that the mRNA levels produced from pC16L2(S) were approximately 30- to 60-fold lower than those produced from pNLCATW (Fig. 4C). The levels of internal control EIAV *gag* mRNA did not vary significantly between the two transfections. The CAT protein levels produced from pC16L2(S) were 140-fold lower than those produced from pNLCATW in the same transfection experiment (Fig. 4C).

Next, cytoplasmic and nuclear RNAs were extracted and analyzed by primer extension. The mRNA levels produced from pC16L2(S) were lower than those produced from pNLCATW in both cellular compartments (Fig. 5A). The results in Fig. 5B show that the mRNA levels produced from pC16L2(S) were 13-fold lower in the cytoplasm and 6-fold lower in the nucleus than those produced from pNLCATW. Analysis of the subcellular distribution of the pC16L2(S) and pNLCATW mRNAs showed that 25 and 41% were cytoplasmic, respectively (Fig. 5). pHCMVtat mRNA was evenly distributed in the cytoplasmic and nuclear compartments (Fig. 5A; 46 and 40% of the pHCMVtat mRNAs were cytoplasmic in the two transfections). The 47S rRNA precursor was found only in the nuclear fractions (Fig. 5A), as expected. CAT protein levels produced from pC16L2(S) in the same transfection experiment were 73-fold lower than those produced from pNLCATW (Fig. 5B). It was observed in all transfection experiments that the inhibitory effect was greater at the protein level than at the mRNA level. These results indicated that the L2 sequence acted by reducing mRNA levels in the nuclear and cytoplasmic compartments and suggested an additional inhibitory effect on mRNA utilization.

**HPV-16 L2 contains cytoplasmic mRNA instability determinants.** To investigate if the decreased levels of L2-containing mRNAs could be explained by a reduced mRNA half-life, HLtat cells were transfected with pC16L2(S) or pNLCATW and treated with actinomycin D for 0, 30, and 60 min. Cytoplasmic RNA was extracted, and mRNA levels were quantified by primer extension. Figure 6A shows that the mRNAs produced from pC16L2(S) were less stable than those produced from pNLCATW, a result which explains, at least in part, the reduced steady-state levels of L2-containing mRNAs. The cytoplasmic half-life of pC16L2(S) mRNAs was 61 min, whereas the pNLCATW-derived mRNAs had a half-life of 161 min (Fig. 6B). The mRNA half-life was reduced approximately threefold when the L2 sequence was present on the mRNA. The pC16L2(S)-derived mRNAs were more stable in the nucleus than in the cytoplasm (data not shown), and we therefore focused on cytoplasmic L2 mRNA. The stability of several cellular mRNAs is affected by translation inhibitors (reviewed in reference 41). To test if CAT-L2 mRNA levels could be induced by translation inhibitors, we treated pC16L2(S)-transfected cells with cycloheximide for 0, 1, 2, and 3 h. Levels of cytoplasmic mRNAs produced from pC16L2(S) continuously increased during the cycloheximide treatment time course (Fig. 7A), indicating that the effect on cytoplasmic mRNA

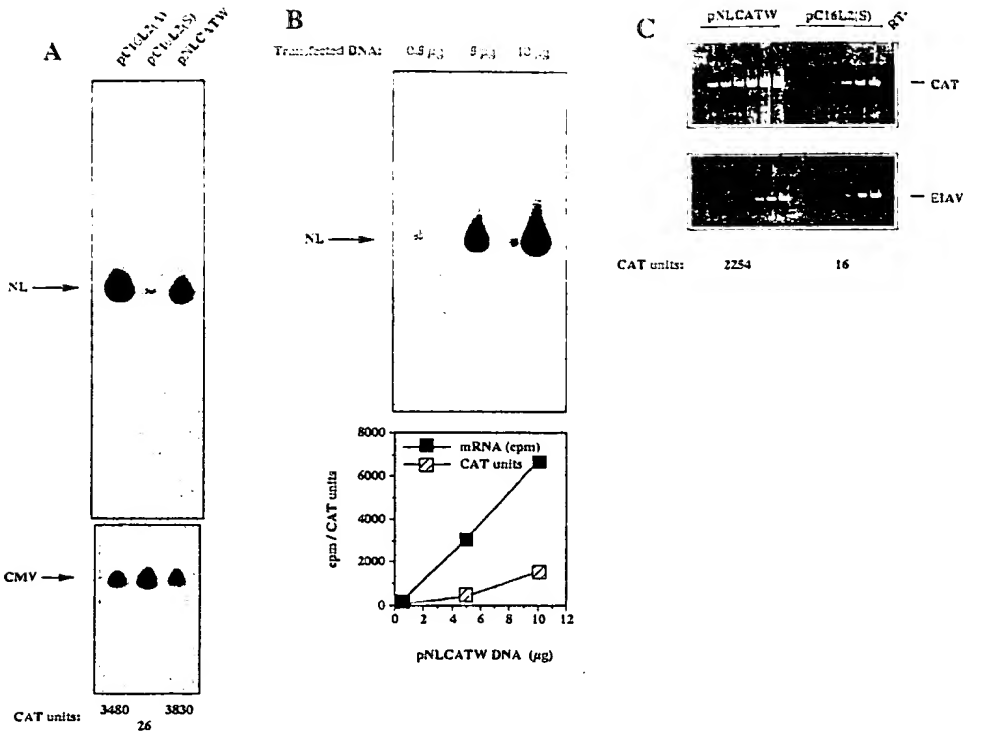


FIG. 4. The HPV-16 L2 inhibitory sequences reduce cytoplasmic mRNA levels. (A) Cytoplasmic mRNA levels produced from the indicated plasmids were detected by primer extension. NL, extension products of mRNAs derived from the HIV-1 LTR promoter; CMV, extension products of the internal control mRNA derived from the CMV immediate-early promoter in pHCMVtat; CAT units, CAT protein levels produced from the indicated plasmids. (B) Serially diluted pNLCATW plasmid DNA was transfected into HLtat cells. Cytoplasmic mRNA levels (counts per minute [cpm]) were quantified by use of a PhosphorImager, and CAT protein levels (CAT units) were quantified by a CAT antigen capture ELISA. NL, primer extension products of mRNAs derived from the HIV-1 LTR promoter in pNLCATW. (C) RT-PCR of fourfold serially diluted cytoplasmic poly(A)$^+$ mRNAs extracted from HLtat cells transfected with plasmid pNLCATW or pC16L2(S) as indicated. The upper panel shows PCR amplification with oligonucleotide primers specific for cDNA generated from the CAT mRNAs produced from pNLCATW and pC16L2(S), and the lower panel shows PCR amplification with oligonucleotide primers specific for cDNA generated from the ElAV *gag* mRNAs produced from the internal control plasmid pE55 (59). RT-, amplification from poly(A)$^+$ mRNA in the absence of reverse transcriptase.

stability was dependent on protein synthesis. There was an approximate fivefold induction after 3 h of cycloheximide treatment (Fig. 7A), while the difference in steady-state mRNA levels between pC16L2(S) and pNLCATW was 10- to 30-fold, indicating that inhibition of translation did not entirely prevent premature cytoplasmic degradation of CAT-L2 mRNAs. To test if specific inhibition of translation of the mRNA produced by pC16L2(S) resulted in increased cytoplasmic mRNA levels, a stable GC-rich hairpin loop that blocks translation of the mRNA was inserted at the 5′ end of pC16L2 (S), generating pC16L2-Stop (Fig. 7B). This plasmid did not produce detectable levels of CAT protein. The mRNA levels produced from pC16L2-Stop were twofold lower than those produced from pC16L2(S) (Fig. 7C). The reason for this may be that the introduction of a stable RNA structure may have effects on the mRNA other than inhibiting translation. These results indicated that the reduction of the cytoplasmic mRNA levels by the L2 sequence was not dependent on translation of the L2-CAT mRNAs. The results presented here also demonstrate that HPV-16 L2 contains a rapid mRNA degradation determinant and suggest that a labile factor targets HPV-16 L2 mRNAs for premature degradation in the cytoplasm.

We wished to confirm that the low cytoplasmic mRNA levels produced from the CAT-L2 hybrid plasmids were a result of the presence of L2 and were not caused by the combination of CAT and L2 sequences. We therefore compared the steady-state cytoplasmic mRNA levels produced from p16L2ΔC (Fig. 7B), designed to express the L2 gene from the HIV-1 LTR promoter in the absence of the CAT gene, with those produced from pC16L2(S). The two plasmids produced similar low cytoplasmic mRNA levels (Fig. 7C), and L2 protein could not be detected in cells transfected with p16L2ΔC (data not shown). In conclusion, the presence of the HPV-16 L2 sequence in the mRNA resulted in decreased mRNA levels, caused by rapid mRNA degradation.

**The cytoplasmic mRNA instability sequence is contained in the 5′-most 845 nt of HPV-16 L2.** To map the negative sequences in HPV-16 L2, we introduced deletions in the L2 sequence contained in pC16L2(S), resulting in plasmids pCL2D, pCL2C, pCL2B, and pCL2A (Fig. 8A). The L2 sequences

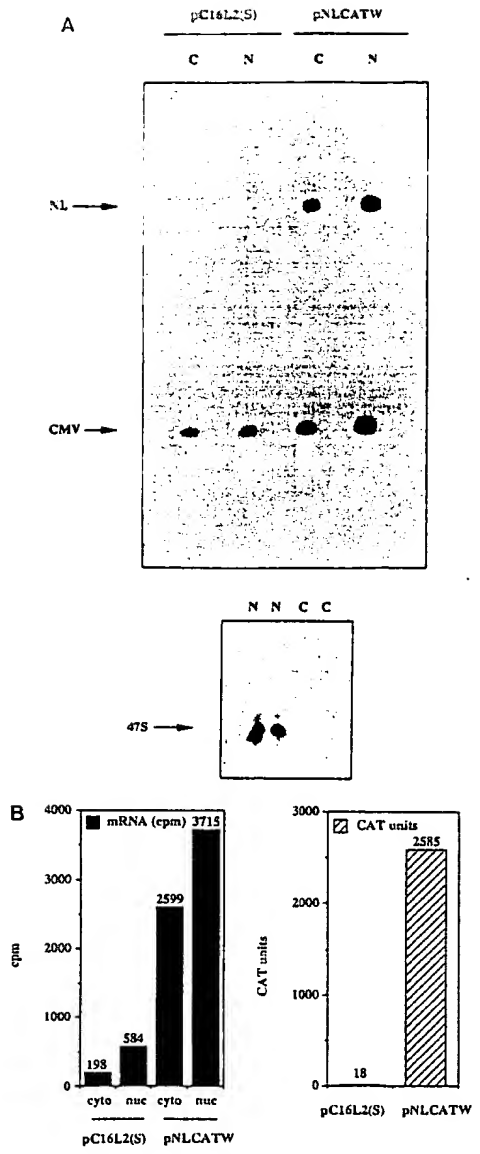


FIG. 5. The HPV-16 L2 inhibitory sequences reduce mRNA levels in both cytoplasmic and nuclear compartments. (A) Cytoplasmic (C) and nuclear (N) mRNA levels produced from the indicated plasmids were detected by primer extension. NL, extension products of mRNAs derived from the HIV-1 LTR promoter; CMV, extension products of the internal control mRNA derived from the CMV immediate-early promoter in pHCMVtat; 47S, extension product of the 47S precursor rRNA. (B) Histogram showing cytoplasmic (cyto) and nuclear (nuc) mRNA levels (counts per minute [cpm]) quantified by use of a PhosphorImager and CAT protein levels (CAT units) produced from the indicated plasmids.

present in pCL2A, pCL2B, and pCL2C inhibited CAT expression when compared to pNLCATW but less so than did the entire L2 sequence present in pC16L2(S) (Fig. 8A). pCL2D produced CAT levels similar to those produced from pNLCATW (Fig. 8A), demonstrating that the inhibitory sequences had been destroyed. Therefore, sequences in the 5′ and 3′ ends of the L2 coding region contributed to the inhibitory activity. Alternatively, inhibitory sequences were located in the 5′ end and in the middle portion of the L2 coding region.

Next, the cytoplasmic mRNA levels produced from pNLCATW and the HPV-16 L2-containing plasmids pC16L2(S), pCL2D, pCL2C, pCL2B, and pCL2A were assayed and quantified by primer extension. pCL2D produced mRNA and CAT protein levels similar to those produced from pNLCATW, demonstrating that the mRNA instability determinant had been destroyed by the deletion. pCL2C was partially inhibitory, indicating that L2 sequences between nt 4228 and nt 4865 were required for the function of the mRNA instability determinant. However, these sequences did not encompass the entire mRNA instability determinant. pCL2B produced mRNA levels comparable to those produced from pC16L2(S), indicating that the first 845 nt contained the complete mRNA instability sequence. To confirm this result, mRNA levels produced from serially diluted pCL2B were determined and compared to mRNA levels produced from pNLCATW and pC16L2(S) (Fig. 8C). The results verified that pCL2B produced low levels of mRNA similar to those produced from pC16L2(S) (Fig. 8C) and that pCL2B and pC16L2(S) produced lower mRNA levels than did pNLCATW (Fig. 8C). Actinomycin D time course experiments with pCL2B- and pNLCATW-transfected cells, followed by analysis of the cytoplasmic mRNA levels produced from these plasmids, showed that the cytoplasmic half-life of pCL2B mRNAs was threefold lower than that of pNLCATW mRNAs (Fig. 8D). These results mapped the HPV-16 L2 mRNA instability sequence to the first 845 nt of the L2 ORF. Interestingly, pCL2A produced higher mRNA levels than did pC16L2(S), but these were lower than those produced from pNLCATW, indicating that the mRNA instability determinant was affected but not destroyed. However, at the protein level, the inhibitory activity of the L2 sequence in pCL2A was stronger than that in pCL2B (Fig. 8B), suggesting the existence of additional inhibitory sequences in the 3′ end of L2.

**Identification of inhibitory sequences in the 3′ end of L2.** To confirm the presence of inhibitory sequences in the 3′ end of L2, sequences spanning various parts of the 3′-most 800 nt of HPV-16 L2 (nt 4865 to nt 5665) were inserted downstream of CAT in pNLCATW. Analysis of the CAT levels produced from pCL2R1 and pCL2R2 (Fig. 9A) revealed that the L2 sequences present in these two plasmids inhibited CAT production to similar extents (Fig. 9A), suggesting that an inhibitory region was located in the middle of these two overlapping sequences. The borders of this region should be nt 5060 and nt 5520. However, the presence of this fragment downstream of CAT, as in pCL2R7, did not result in strong inhibition (Fig. 9A), indicating that additional sequences in the 5′ and 3′ ends were required for efficient inhibition. This suggestion was consistent with larger deletions of 5′ and 3′ sequences, as in pCL2R5 and pCL2R4, respectively, resulting in a loss of inhibition (Fig. 9A). We concluded that an inhibitory region was located downstream of nt 4860 in the L2 coding sequence and that efficient inhibition required the presence of 500 to 600 nt of the L2 3′ end.

Next we monitored the cytoplasmic mRNA levels produced from plasmids pNLCATW, pCL2R1, and pCL2R4 (Fig. 9A). Primer extension revealed that pCL2R1 produced approximately twofold-lower cytoplasmic mRNA levels than did

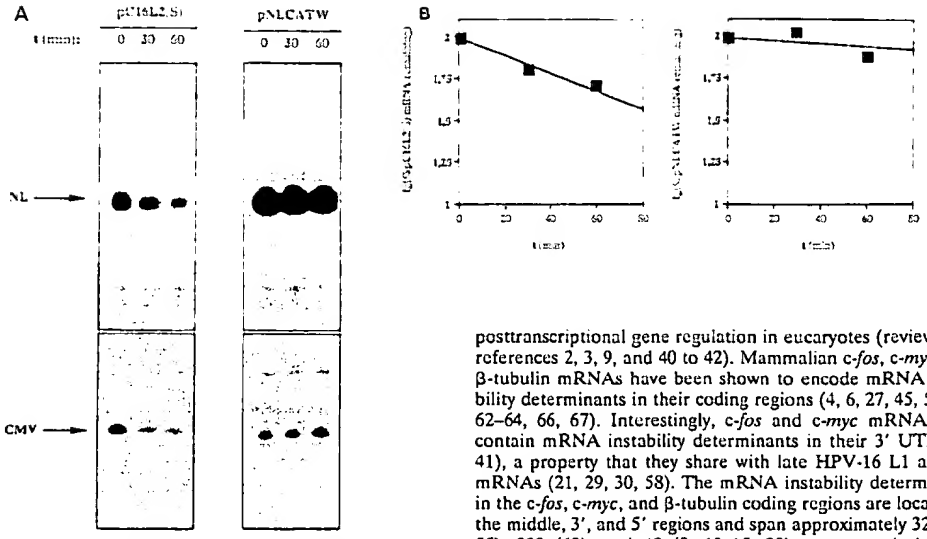


FIG. 6. The HPV-16 L2 inhibitory sequences reduce cytoplasmic mRNA stability. (A) Cytoplasmic mRNA levels produced from the indicated plasmids were detected by primer extension at different times [t (min)] after the addition of 10 μg of actinomycin D (Sigma) per ml to the media. NL, extension products of mRNAs derived from the HIV-1 LTR promoter; CMV, extension products of the internal control mRNA derived from the CMV immediate-early promoter in pHCMVtat. (B) Quantitation of mRNA levels at the indicated times by use of a PhosphorImager after normalization to the internal control mRNA levels. lg, log. A representative experiment is shown.

pCL2R4 and pNLCATW (Fig. 9B). The mRNAs produced from these plasmids were of the expected sizes when analyzed by Northern RNA blotting (Fig. 9C). These results indicated that the 12-fold inhibitory effect conferred by the L2 sequence in pCL2R1 (Fig. 9A) was at the posttranscriptional level. The presence of the 3'-most 800 nt of the L2 sequence upstream of the transcribed region, as in plasmids pL2HU(S) and pL2HU(A) (Fig. 9A), had no effect on CAT levels (Fig. 9A), further indicating that inhibition occurred posttranscriptionally. We concluded that weaker inhibitory sequences were present in the 3'-most 800 nt of L2. These inhibitory sequences acted independently of those in the 5' end of L2.

## DISCUSSION

Here we show that the HPV-16 L2 coding region contains *cis*-acting inhibitory sequences that act in an orientation-dependent manner to reduce cytoplasmic and nuclear mRNA levels. The low cytoplasmic mRNA steady-state levels were a result of the reduced stability of mRNAs containing L2. Mapping experiments demonstrated that the 5' end of the L2 coding region contained an mRNA destabilization sequence and that the 3' end of L2 contained a weaker inhibitory sequence that acted posttranscriptionally to reduce protein levels. Furthermore, we showed that strong inhibitory sequences in the L1 and L2 coding regions are found in HPV-16 but not in HPV-1, suggesting that late-gene expression is differently regulated by these two HPV types.

Regulation of mRNA stability is a common mechanism of posttranscriptional gene regulation in eucaryotes (reviewed in references 2, 3, 9, and 40 to 42). Mammalian c-*fos*, c-*myc*, and β-tubulin mRNAs have been shown to encode mRNA instability determinants in their coding regions (4, 6, 27, 45, 54, 55, 62–64, 66, 67). Interestingly, c-*fos* and c-*myc* mRNAs also contain mRNA instability determinants in their 3' UTRs (9, 41), a property that they share with late HPV-16 L1 and L2 mRNAs (21, 29, 30, 58). The mRNA instability determinants in the c-*fos*, c-*myc*, and β-tubulin coding regions are located in the middle, 3', and 5' regions and span approximately 320 (54, 55), 320 (63), and 40 (8, 13–15, 38) nt, respectively. The HPV-16 L2 coding region mRNA destabilizing determinant is contained within the first 845 nt (Fig. 8). Mapping of the 5' end of the L2 sequence will show if the size of the functional element is smaller. The L2 coding region does not contain AUUUA or UUUUU motifs, which are commonly found in AU-rich mRNA instability elements (9, 41), but does have a 60% A+U content, similar to that of an mRNA instability sequence located in the HIV-1 *gag* ORF (50). The human insulinlike growth factor II mRNA contains an RNA cleavage-promoting sequence in the 3' UTR, consisting of two elements located approximately 2,000 nt apart (44). These RNA elements hybridize to form the functionally active stem-loop structure (44). It remains to be investigated if it is the RNA primary or secondary structure that is the major determinant of the activity of the HPV-16 L2 mRNA instability determinant.

It was previously concluded that translation of the c-*myc* and c-*fos* mRNAs specifically was required for deadenylation and rapid degradation (27, 45, 64). The function of the negative element in the β-tubulin coding region is dependent on the production of the first amino acids of the β-tubulin protein (66, 67). Furthermore, the yeast *MATα1* mRNA is rapidly degraded as a result of the presence in the mRNA coding region of rare codons (7, 26). Similarly, we showed that treatment of cells with the translation elongation inhibitor cycloheximide rendered the CAT-L2 hybrid mRNAs more stable in the cytoplasm (Fig. 7A). However, the function of the mRNA instability sequence in the CAT-L2 hybrid mRNAs was not dependent on translation of these mRNAs specifically (Fig. 7B and C), suggesting that a labile factor targets the L2 mRNA for rapid degradation. This suggestion is not without precedent, since premature degradation of a c-*fos* 3' UTR-containing mRNA was shown to be inhibited by cycloheximide but not by insertion into the mRNA of sequences that blocked the translation of c-*fos* (31). This idea is consistent with our observations of reduced CAT-L2 mRNA levels in both the cytoplasmic and the nuclear compartments (Fig. 5), suggesting that mRNA destabilization occurs in the nucleus and the cytoplasm. Alternatively, inhibition of translation by cycloheximide affects the

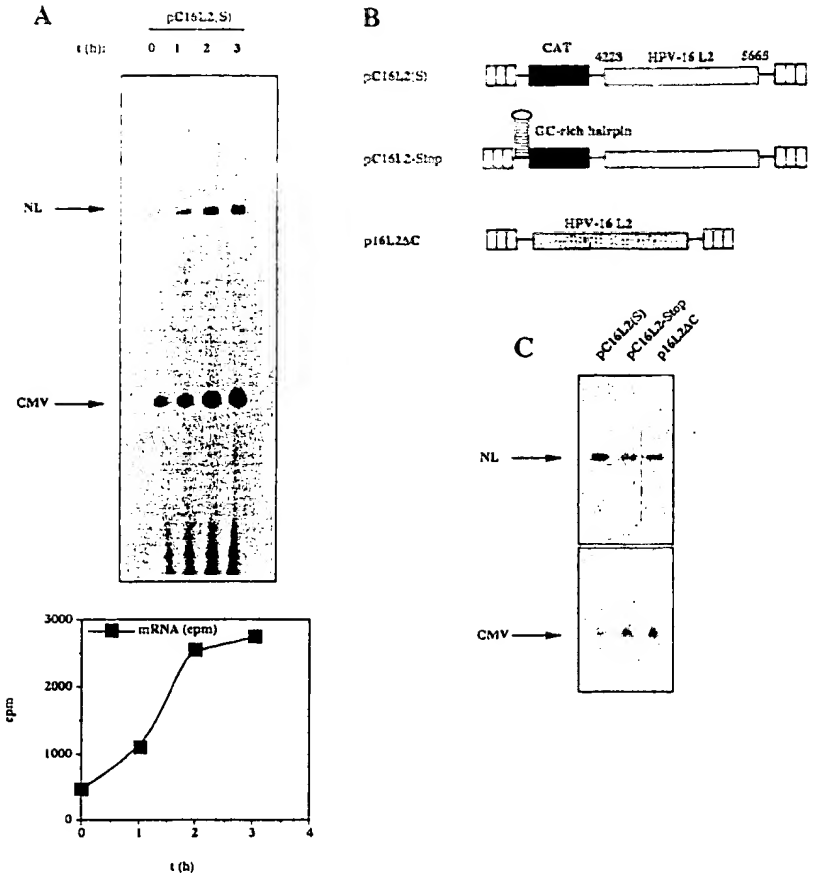


FIG. 7. (A) The effect on cytoplasmic mRNA levels of the HPV-16 L2 inhibitory sequences could be reduced by translation inhibitors. Cytoplasmic mRNA levels produced from the indicated plasmids were detected by primer extension at different times [t (h)]) after the addition of 10 μg of cycloheximide (Sigma) per ml to the cell culture media. NL, extension products of mRNAs derived from the HIV-1 LTR promoter; CMV, extension products of the internal control mRNA derived from the CMV immediate-early promoter in pHCMVtat. The graph shows quantitation of the mRNA levels at the indicated times by use of a PhosphorImager after normalization to the internal control mRNA levels. A representative experiment is shown. (B) Translation of the CAT-L2 mRNA specifically is not required for the reduction of cytoplasmic mRNA levels. Plasmid structures are shown. Shaded boxes represent the HPV-16 L2 coding region, numbers indicate nucleotide positions (51), striped boxes indicate HIV-1 LTRs, black boxes indicate the CAT gene, and a GC-rich hairpin is shown as a stem-loop structure. Plasmid names are indicated on the left. (C) Cytoplasmic mRNA levels produced from the indicated plasmids were detected by primer extension. NL, extension products of mRNAs derived from the HIV-1 LTR promoter; CMV, extension products of the internal control mRNA derived from the CMV immediate-early promoter in pHCMVtat.

stability of the mRNA only in the cytoplasm. The c-*fos* coding region instability determinant interacts with cellular factors (10), and the c-*myc* mRNA instability determinant binds a 70-kDa protein which protects against degradation of the c-*myc* mRNA (39). A recent report indicates that the HPV-16 L2 protein interacts nonspecifically with nucleic acids (69). However, production of the HPV-16 L2 protein is most likely not required for the inhibitory activity of the L2 mRNA. It remains to be investigated by what mechanism L2-containing mRNAs are degraded in the cytoplasm and the nucleus and if cellular proteins bind to them. We cannot exclude the possibility that L2-containing mRNAs are retained in the nucleus, where they are prematurely degraded.

HPVs infect dividing cells in the basal cell layer of the stratified epithelium. As the infected cell moves toward the upper cell layers and differentiates, HPV late-gene expression is activated (11, 23, 28, 32, 33, 56). This differentiation-dependent HPV late-gene expression pattern has been observed for several different HPV types, illustrating that the expression of late genes is suppressed in the lower cell layers, while in the upper strata, with differentiated cells, this block is relieved. Interestingly, the expression of c-*fos* in squamous cell epithelium also is differentiation dependent, with higher c-*fos* mRNA and protein levels in the upper strata (19). Therefore, the inhibitory activity of the c-*fos* and HPV-16 L2 coding region determinants must be relieved as the cell differentiates. Inter-

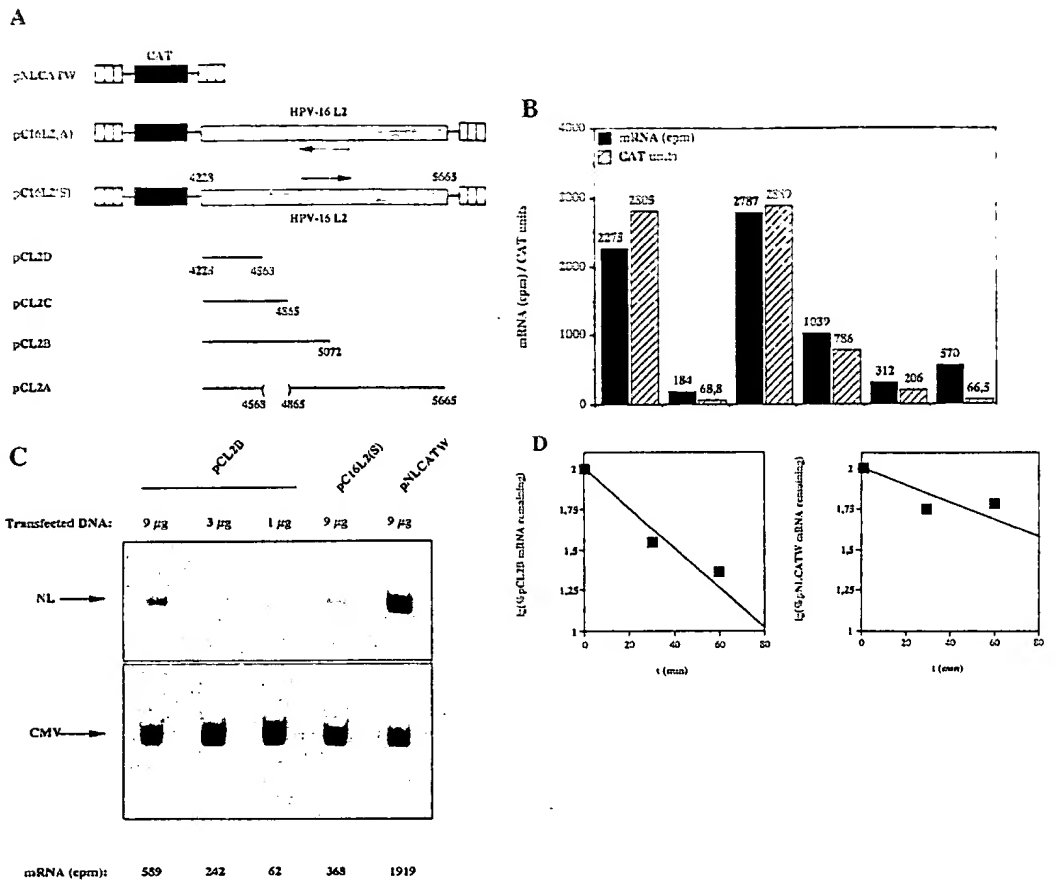


FIG. 8. The 5' end of the L2 sequence contains a cytoplasmic mRNA instability determinant. (A) Schematic structures of CAT expression plasmids. Striped boxes indicate HIV-1 LTRs, black boxes represent the CAT gene, shaded boxes represent the HPV-16 L2 coding region, and arrows indicate antisense (←) and sense (→) orientations. Numbers refer to nucleotide positions in the HPV-16 genomic clone (51). Plasmid names are shown on the left. The depicted plasmids were transfected in triplicate into HLtat cells, and CAT levels were monitored in a CAT antigen capture ELISA and normalized to SEAP levels produced from the internal control plasmid pCS1X (58). The mean CAT values obtained after triplicate transfections with pNLCATW (58) were divided by the mean CAT values obtained after triplicate transfections with the indicated plasmids to yield fold inhibition (for plasmids from top to bottom, fold inhibition was 1, 1.2, 88, 1, 9, 14, and 56). (B) Histogram showing the cytoplasmic mRNA levels quantified by use of a PhosphorImager (counts per minute [cpm]) and CAT protein levels (CAT units) produced from plasmids pNLCATW, pC16L2(S), pCL2D, pCL2C, pCL2B, and pCL2A. (sets of bars from left to right). (C) Serially diluted pCL2B plasmid DNA was transfected as indicated. Cytoplasmic mRNA levels (cpm) were quantified by use of a PhosphorImager. NL, primer extension products of mRNAs derived from the HIV-1 LTR promoter; CMV, extension products of the internal control mRNA derived from the CMV immediate-early promoter in pHCMVtat. (D) Cytoplasmic mRNA levels produced from the indicated plasmids were detected by primer extension at different times [t (min)] after the addition of 10 µg of actinomycin D (Sigma) per ml to the media. NL, extension products of mRNAs derived from the HIV-1 LTR promoter; CMV, extension products of the internal control mRNA derived from the CMV immediate-early promoter in pHCMVtat. The graphs show quantitation of the mRNA levels at the indicated times by use of a PhosphorImager after normalization to the internal control mRNA levels. lg, log. A representative experiment is shown.

estingly, c-*myc* production decreases as myoblasts differentiate. A recent report indicated that this effect is attributable to the c-*myc* coding region mRNA instability determinant (65), demonstrating an inverse correlation between the inhibitory activity of the RNA sequence and cell differentiation for c-*fos* and HPV-16 late mRNAs. It remains to be investigated if the inhibitory sequences in HPV-16 L1 and L2 are as active in terminally differentiated cells as in dividing cells.

An attempt to classify HPVs into different groups based on the amount of virus present in benign lesions has been made and has suggested that HPVs can be divided into three groups: productive, weakly productive, and nonproductive (57). It is of interest to note that HPV-1 categorically falls into the productive group, characterized by the detection of moderate to large amounts of virus in lesions, whereas HPV-16 belongs to the weakly productive group, where only minute amounts of virus can be detected. Since we have identified strong negative sequences in HPV-16 L1 and L2 (58; this study) but not in

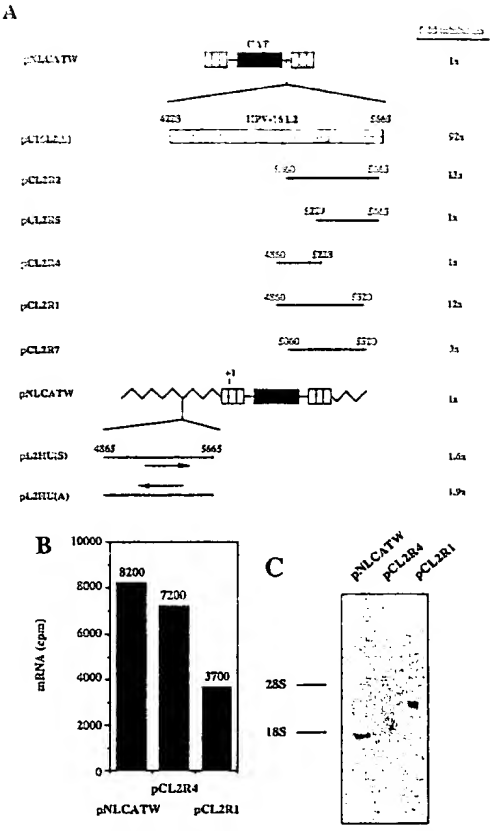


FIG. 9. The 3' end of L2 contains weak inhibitory sequences. (A) Schematic structures of CAT expression plasmids. Striped boxes indicate HIV-1 LTRs, black boxes represent the CAT gene, shaded boxes represent the HPV-16 L2 coding region, and thick black lines indicate subfragments of the L2 coding region. Numbers refer to nucleotide positions in the HPV-16 genomic clone (51). Arrows indicate sense (→) and antisense (←) orientations. Jagged line, vector sequence; +1, start of transcription in the HIV-1 LTR promoter. Plasmid names are shown on the left. The depicted plasmids were transfected in triplicate into HLtat cells, and CAT levels were monitored in a CAT antigen capture ELISA and normalized to SEAP levels produced from the internal control plasmid pCS1X (58). The mean CAT values obtained after triplicate transfections with pNLCATW (58) were divided by the mean CAT values obtained after triplicate transfections with the indicated plasmids to yield fold inhibition. (B) Cytoplasmic mRNA levels produced from the indicated plasmids were detected by primer extension. The histogram shows the mRNA levels quantified by use of a PhosphorImager (counts per minute [cpm]). (C) Northern RNA blotting of cytoplasmic RNA extracted from cells transfected with the indicated plasmids. The positions of the 28S and 18S rRNA species are shown.

HPV-1 L1 and L2, it is reasonable to speculate that the negative sequences present in the coding regions may explain the different amounts of virions present in lesions caused by HPV-1 and HPV-16. In contrast, both HPV-1 and HPV-16 show strict cell differentiation-dependent expression of late genes. This property correlates with the presence of a negative element in the 3' UTRs of the late mRNAs of both HPV types (29, 30, 58, 60), suggesting that the block in late-gene expression caused by the 3' UTR element is alleviated as the cell differentiates. It would be of interest to determine the effect of cell differentiation on the activity of the 3' UTR and coding region inhibitory sequences.

The regulation of viral late-gene expression caused by the presence of negative regulatory sequences on the mRNAs coding for structural proteins appears to be a common strategy used by many viruses. Although such inhibitory RNA sequences have not yet been characterized in detail, several reports on negative sequences on viral late mRNAs have been presented. For example, HIV-1 contains inhibitory sequences in the Gag, Pol, and Env coding regions (16, 34, 37, 46, 47, 50) and human T-cell leukemia virus type 1 contains inhibitory sequences in the 5' UTR (52) and in the Pol and Env coding regions (43). An mRNA instability sequence previously identified in the HIV-1 *gag* ORF was shown to reduce mRNA levels in both cytoplasmic and nuclear compartments (50), similar to the data presented here for HPV-16 L2 (Fig. 5). The HIV-1 *gag* mRNA inhibitory element interacted specifically with poly(A)-binding protein 1 (1), but in preliminary experiments we did not detect binding to the L2 sequences of proteins with an affinity for poly(A).

The presence of multiple inhibitory sequences on viral late mRNAs encoding structural proteins appears to be a common property of HIV-1, HTLV-1, and HPV-16. For example, HIV-1 contains inhibitory sequences in the Env coding region (12, 37) that would be present on the Env-producing and Gag-Pol-producing mRNAs as well as inhibitory sequences in the Gag and Pol coding regions (16, 34, 47, 50). A similar arrangement is observed for HPV-16, where the HPV-16 L1 coding region contains inhibitory elements (58) that would be present on both the L1 mRNAs and the L2-L1 mRNAs (5). In addition to these negative elements, the L2-L1 mRNAs would contain the L2 coding region inhibitory sequences described here. Therefore, the inhibitory sequences in HPV-16 L2 would allow independent regulation of L1 and L2 production. Perhaps the presence of inhibitory sequences in the L2 coding region allows a balanced production of L1 and L2 and reflects a requirement for the production of a certain ratio between L1 and L2 molecules to generate correctly assembled virions. Alternatively, perhaps the posttranscriptional processing and regulation of expression of a polycistronic mRNA are different from those of a monocistronic mRNA and therefore require more complex *cis*-acting signals. In conclusion, the presence of negative elements on viral late mRNAs allows the virus to regulate late-gene expression and virus production. This ability may be of critical importance for the virus in avoiding host immune system surveillance and in establishing persistent infections. Subgenomic virus expression plasmids encoding late genes lacking negative sequences may be valuable tools for the development of DNA vaccines.

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# Analysis of Human Papillomavirus Type 16 Late mRNA 3' Processing Signals In Vitro and In Vivo

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**In the human papillomavirus type 16 genome, three late mRNA putative 3' processing signals, designated LP1, LP2, and LP3, are located downstream of the late coding region. Our results show, both in vitro and in vivo, that in HeLa cells, the LP2 signal functions. Thus, the restriction in human papillomavirus type 16 late-gene expression observed in HeLa cells and other nondifferentiated epithelial cells is not achieved by regulation of late mRNA poly(A) site usage. Interestingly, alteration of three nucleotides in the GU-rich downstream sequence element converts the nonfunctional LP1 to an efficient 3' processing site, suggesting that LP1 may function in cell types other than HeLa, such as differentiated keratinocytes. Our transfection studies have identified a negative regulatory element located immediately upstream of the late mRNA 3' processing signals; this element was not associated with any alteration in 3' processing and may act as an mRNA instability element.**

Papillomaviruses, containing double-stranded circular DNA genomes of approximately 8 kilobases, are the etiological agents of warts or papillomas and infect humans and animals with strict host and tissue specificity (reviewed in reference 2). The human papillomaviruses (HPV) are diverse, consisting of at least 60 distinct types, some of which have been associated with malignancy; HPV-5, -8, and -14 are linked with malignant skin lesions in patients with epidermodysplasia verruciformis (14), and HPV-16, -18, -31, -33, -35, -39, -45, -51, and -52 are linked with cervical cancer and other ano-genital cancers (reviewed in reference 3).

Research on papillomavirus-host cell interactions is hindered by the lack of a tissue culture system capable of supporting virus growth; virus capsid antigens and virus particles are observed only in cells that are in the process of keratinization or are already keratinized (21). Virus DNA replication and capsid protein synthesis have not been detected in basal keratinocytes. The nature of this restriction in undifferentiated cells is not known and is of considerable importance in understanding transmission of these viruses. Here we have utilized HeLa cells, a human cervical carcinoma cell line, which probably most closely resemble basal keratinocytes and which are nonpermissive for late-gene expression. We describe the use of in vitro polyadenylation assays and in vivo transfection assays to examine the HPV-16 sequences involved in late mRNA 3' end formation and to investigate the possibility that regulation of HPV-16 late-gene expression may be achieved through differential usage of the late poly(A) site.

Analysis of the published HPV-16 DNA sequence (26) indicates that the prototype HPV-16 noncoding region (NCR) contains three putative late mRNA 3' processing sites (LP1, LP2, and LP3) which, in RNA, consist of a conserved AAUAAA signal (22) and downstream sequences. For many 3' processing sites, a GU-rich downstream element is essential and a consensus YGUGUUYY sequence (where Y is a pyrimidine) has been described (16). Two of these sites (LP1 and LP2) are located within 210 base pairs (bp) of the L1 open reading frame stop codon (at 7152), whereas LP3 is approximately 550 bp downstream of the stop codon (Fig. 1). In addition, we have a naturally occurring variant, HPV-16del (12), with a deletion of 122 bp in the NCR, having endpoints in a direct repeat, UGUUUU, at positions 7278 and 7400; this deletion removes a portion of the downstream sequence of LP1 and the whole of LP2 but leaves LP3 intact (Fig. 1).

**LP2 functions efficiently in extracts from HeLa cells and is the only functional late mRNA 3' processing site in vitro.** The putative late 3' processing signals from the prototype and HPV-16del genomes were examined by using 3' processing in vitro (19). Precursor RNAs, synthesized from recombinant pGEM plasmids by using SP6 or T7 RNA polymerase (18) and containing the appropriate HPV-16 sequences, were incubated with HeLa cell nuclear extracts (4, 19) in the presence of ATP or 3' dATP (17, 19). In reactions containing ATP, precursor RNA PD753, which contained HPV-16 sequences from the *PstI* site at 7008 to the *DdeI* site at 7761 (Fig. 2B) and in which all three signals, LP1, LP2, and LP3, were present, was efficiently polyadenylated (Fig. 3A). In the presence of 3' dATP, which inhibits polyadenylation but does not prevent cleavage, with PD753 RNA, a prominent cleavage product (CP2) of approximately 355 nucleotides was detected; this corresponded to cleavage at the LP2 site (Fig. 3A). A second precursor RNA, PD753del, which lacks LP2, was not cleaved at either the LP1 or the LP3 site (Fig. 3A); the faint band at a size slightly larger than CP1 does not represent LP1 cleavage, since it is also present in the ATP reaction.

To eliminate any potential effects of the upstream LP1 and LP2 signals on LP3, we examined the use of LP3 in isolation by preparing a precursor RNA, ED308, which contained HPV-16 sequences from the *EcoRI* site at 7453 to the *DdeI* site at 7761 (Fig. 2B). ED308 RNA was not cleaved at the LP3 site (Fig. 3A), indicating that this site is inactive on its own. Lack of LP3 utilization may be due to the absence of a downstream element which closely resembles the GU-rich consensus.

Unlike the LP3 putative site, LP1 contains sequences with strong homology to both the AAUAAA and GU-rich elements required for RNA processing. To investigate the

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                P  T  T  S  S  T  S  T  T  A     K  R  R     R  R  K  L
7101  CCCACCACCT CATCTACCTC TACAACTCCT AAACGCAAAA AACGTAAGCT
      L1 stop
7151  GTAAGTATTG TATGTATGTT CAATTACTGT TGTTTGTTGT GTATATGTTT
7201  GTATGTGCTT GTATGTGCTT GTAATATTTA AGTTGTATGT GTGTTTGTAT
                pA signal (LP1)                   YGTGTTYY
7251  GTATGGTATA ATAAACAGGT GTGTATGTGT TTTTAAATGC TTGTGTAACT
                      pA signal (LP2)             YG
7301  ATTGTGTCAT GCAACATAAA TAAACTTATT GTTTCAACAC GTACTAATTG
      TGTTTT
7351  TGTTGTGGTT ATTCATTGTA TATAAACTAT ATTTGCTACA TCCTGTTTTT
7401  GTTTTATATA TACTATATTT TGTAGCDCCA GCCCCATTTT GTAGC.....
7451  .....TTACA CTGCACTATG TGCAACTACT GAATCACTAT GTACATTGTG
                pA signal (LP3)                   YGTGTTYY
7501  TCATATAAAA TAAATCACTA TGCGCCAACG CCTTACATAC CCCTGTTAGG
```

FIG. 1. Sequence of HPV-16 DNA in the genome region containing the putative late mRNA 3' processing signals. The HPV-16 DNA sequence is taken from Seedorf et al. (26). Positions of the L1 stop codon, poly(A) signals, and YGTGTTYY consensus for LP1, LP2, and LP3 are indicated. Arrows indicate the approximate position of the endpoints, within the direct repeat TGTTTT, for the 122-bp deletion in HPV-16del.

preferential utilization of LP2 over LP1, we examined poly(A) site use in isolation. Double-stranded oligonucleotides, consisting of sequences from LP1 (a 73-mer containing HPV-16 sequences from positions 7248 to 7315) and LP2 (a 67-mer containing HPV-16 sequences from positions 7305 to 7367), were synthesized and cloned into pGEM-1, and precursor RNAs produced from these plasmids were analyzed in vitro. Results (Fig. 3B) show that LP1 RNA gave a barely detectable CP1 cleavage product and was poorly polyadenylated. By contrast, LP2 RNA was efficiently cleaved and polyadenylated to a level comparable to that observed with the complete sequence (compare with Fig. 3A). Constructs in which the LP1 and LP2 synthetic sequences were linked in tandem were made. Precursor LP1/LP2 RNA was preferentially cleaved at the LP2 site (Fig. 3B). Similar results were obtained for an RNA precursor with the order LP2/LP1 (data not shown). These data clearly indicate that LP2 functions efficiently with extracts from HeLa cells and is the only functional late mRNA 3' processing site in vitro. The preferential usage of LP2 over LP1 is a feature of RNA sequences at these sites and is independent of their order in a precursor.

**Lack of LP1 utilization is due to a poorly functional downstream GU-rich element.** Lack of LP1 utilization was intrinsic to the LP1 site, and one possibility considered was that a poorly functional downstream GU-rich element was responsible. Comparison of the GU-rich elements of LP1 and LP2 indicated that they differed at the last three nucleotides. Substitution of an AAC with UGU would convert the LP1 GU-rich element to a sequence identical to a GU-rich element (UGUGUUGU) present within LP2 at a similar spacing from the poly(A) signal. A double-stranded LP1mut oligonucleotide with these base changes was cloned into pGEM-1. Precursor LP1mut RNA from this plasmid was efficiently cleaved at approximately 15 to 20 nucleotides from the AAUAAA signal, which is similar to that observed with LP2 RNA (Fig. 3B). These results indicate that the lack of LP1 utilization is due to a poorly functional downstream GU-rich element.

**Transfection studies indicate the presence of a negative regulatory element upstream of the functional LP2 site.** These putative late mRNA 3' processing signals were examined further in vivo by insertion of appropriate HPV-16 DNA sequences, as shown in Fig. 2, into the CAT expression vector pLW1 (6), which lacks poly(A) site sequences. CAT levels were determined (8, 25) following transfection of these recombinants into HeLa cells (9). Recombinants CAT PP1775 and CAT PP1775del containing prototype and HPV-16del sequences between the *Pst*I sites at positions 7008 and 879 were analyzed; CAT PP1775 contains LP1, LP2, and LP3, while CAT PP1775del contains LP1 and LP3. The

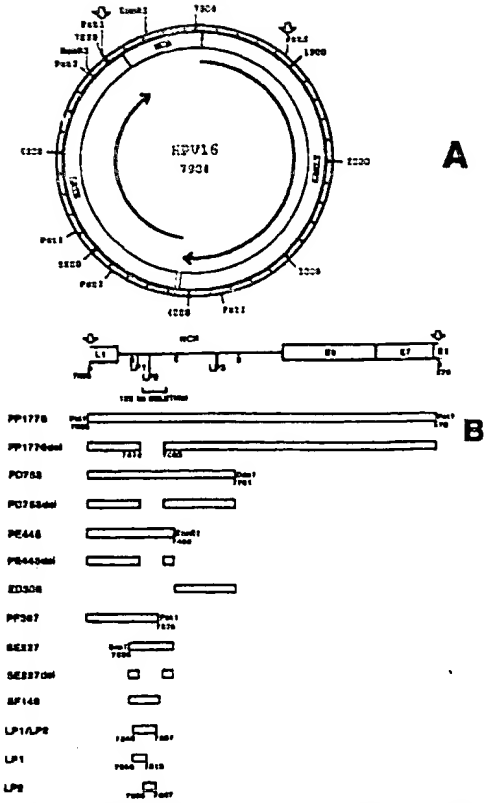


FIG. 2. HPV-16 sequences analyzed by transfection or 3' processing studies. (A) Organization of the HPV-16 genome, with the positions of the early region, the late region, and the NCR indicated. Marked are the *Pst*I sites, and arrows indicate the endpoints of the PP1775 fragment (positions 7008 to 879). Below is shown an enlargement of the HPV-16 PP1775 fragment with the positions of the L1, E6, E7, and E1 open reading frame sequences and NCR indicated. Marked are the positions of the putative late mRNA 3' processing sequences LP1, LP2, and LP3 and various restriction enzyme cleavage sites (P = *Pst*I, S = *Sty*I, E = *Eco*RI, and D = *Dde*I). Brackets locate the position of the 122-bp deletion in the HPV-16del genome between positions 7278 and 7400. (B) Relative positions of PP1775, PP1775del, and respective subfragments which have been analyzed by transfection or 3' processing studies.

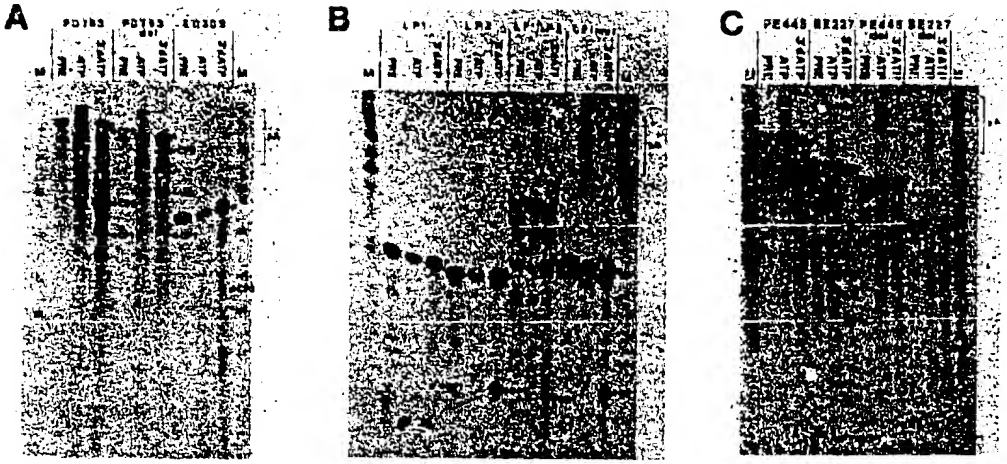


FIG. 3. In vitro 3' processing with precursor RNAs containing putative late mRNA 3' processing signals. Precursor RNAs examined were as follows. (A) PD753, which contains all three signals, LP1, LP2, and LP3; PD753del, which contains LP1 and LP3; and ED308, which contains LP3 only. (B) LP1; LP2; LP1mut, which has a substitution of three nucleotides (UGU instead of AAC) in the downstream GU-rich element; and LP1/LP2, which has tandem LP1 and LP2 sequences. (C) PE445 and PE445del, which contain the negative regulatory sequences, and SE227 and SE227del, which lack these sequences. Analysis of RNA products was performed on 8% denaturing polyacrylamide gels. PRE, Unincubated precursor RNA; ATP, polyadenylation reaction containing ATP; 3' dATP, polyadenylation reaction containing 3' dATP; M, RNA size markers. Positions of polyadenylated RNA (pA), the full-length precursor RNA (P), and the 5' portion of the RNA cleaved at the LP1 poly(A) site (CP1), the LP2 poly(A) site (CP2), and the LP3 poly(A) site (CP3) are indicated.

HPV-16 fragments used also contain additional late, early, and NCR sequences. Surprisingly, both of these constructs failed to produce CAT activity (Fig. 4, lanes 1 and 2), even though CAT PP1775 contained LP2, which functions in vitro. This suggested the presence of inhibitory sequences within this *PstI* fragment which prevented CAT expression. To identify any inhibitory sequences, subfragments (Fig. 2B) of the *PstI* fragment were inserted into pLW1 and the CAT activities from these constructs were determined. Removal of HPV-16 sequences downstream of the *EcoRI* site at position 7453 (CAT PE445 and CAT PE445del), which takes away a large portion of the NCR, including LP3 and the E6, E7, and E1 open reading frame sequences, did not increase CAT activity (Fig. 4, lanes 3 and 4, respectively). By contrast, subsequent removal of sequences upstream of the *SspI* site at position 7226, which removes the L1 open reading frame sequences and most of the 3' untranslated region (plasmid CAT SE227), conferred high-level CAT activity to this recombinant containing prototype HPV-16 sequences. However, the recombinant CAT SE227del, which contains HPV-16del sequences and lacks LP2 sequences, also produced CAT activity, but levels were considerably lower than with CAT SE227 (Fig. 4, compare lanes 5 and 6). These data, summarized in Table 1, indicate that sequences between the *PstI* site at 7008 and the *SspI* site at 7226 exert a negative regulatory effect on mRNA expression from the CAT constructs. Similar results were obtained with Cos1 cells, implying that the HPV-18 genomes present in HeLa cells (24) are not involved in the negative regulatory effect of the HPV-16 sequences.

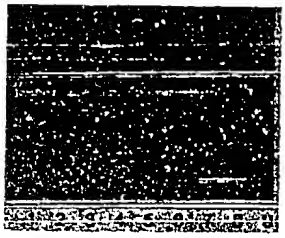

FIG. 4. CAT levels in HeLa cell extracts transfected with the HPV-16/CAT plasmid series. Transfections, containing 10 μg of test plasmid, were performed as described previously (9). Cell extracts were prepared, and CAT activity was assayed by the procedure of Gorman et al. (8). Lanes: 1, CAT PP1775; 2, CAT PP1775del; 3, CAT PE445; 4, CAT PE445del; 5, CAT SE227; 6, CAT SE227del; 7, pLW1; 8, pTER5; and 9, no DNA. pTER5 is essentially pLW1 with the HSV-2 IE 5 mRNA 3' processing signal inserted downstream of the CAT gene (16).

LP2 appears to be the functional poly(A) site in vivo, since CAT SE227del, which lacks LP2, gave CAT levels only 2.7% of those given by CAT SE227, which contains LP2. To confirm this, the synthetic LP1, LP2, and LP1mut sequences were inserted separately into pLW1 downstream of the CAT gene and were transfected into HeLa cells. Results (Table 1) indicate that recombinant CAT LP2 gave high CAT activity, and levels obtained were very similar to those obtained with CAT SE227, which contains LP1 and LP2 but lacks the negative regulatory element. Recombinant CAT LP1 also gave some CAT activity, and levels obtained were 11.4% of those with the CAT SE227 recombinant, whereas the in vitro analysis of LP1 RNA gave negligible cleavage. The recombinant containing LP1mut sequences gave CAT activity

TABLE 1. CAT activities obtained with the HPV-16/CAT plasmid series[a]

| Plasmid | CAT levels | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | % SE227 |
| CAT PP1775 | 0.2 | 3.6 | 0.5 | 0.3 | 1.1 | 0.6 |
| CAT PP1775del | 0.1 | 0.7 | 0.6 | 0.2 | 0.4 | 0.2 |
| CAT PE445 | 0.6 | 0.3 | 1.2 | 1.2 | 0.8 | 0.4 |
| CAT PE445del | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| CAT SE227 | 152.2 | 216.4 | 132.8 | 254.1 | 189.9 | 100.0 |
| CAT SE227del | 9.0 | 2.3 | 5.1 | 4.1 | 5.1 | 2.7 |
| CAT LP1 | 12.2 | 36.5 | 29.8 | 7.4 | 21.5 | 11.4 |
| CAT LP2 | 172.2 | 168.4 | 182.3 | 252.7 | 193.9 | 102.6 |
| CAT LP1mut | 177.1 | 108.6 | 159.9 | 166.0 | 152.9 | 80.9 |
| pLW1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| pTER5 | 481.4 | 566.7 | 107.4 | 135.6 | 326.8 | 173.0 |
| HeLa | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

[a] Procedures were carried out as described in the legend to Fig. 4, except that CAT assays were performed by the method of Seed and Sheen (25). Assays of four independent experiments are shown, and values for each experiment are expressed as picomoles of chloramphenicol acylated per microgram of protein per hour. Mean values were used to indicate percent CAT levels relative to that of CAT SE227. The positive control plasmid pTER5 is essentially pLW1 with the HSV-2 IE 5 mRNA 3' processing signal inserted downstream of the CAT gene (16).

similar to that observed with recombinants CAT LP2 and CAT SE227, and levels were approximately eightfold greater than those of CAT LP1. These data confirm that LP2 functions in vivo and indicate that alteration of three nucleotides in the LP1 downstream GU-rich element makes this a much more efficient poly(A) site in vivo.

**Negative regulatory elements not associated with any alteration in RNA 3' processing.** To investigate the effects of the negative regulatory element on RNA 3' processing, we compared levels of 3' processing in vitro by using precursor RNAs containing (PE445) or lacking (SE227) the negative regulatory sequences. Both RNAs are efficiently cleaved and polyadenylated at the LP2 site (Fig. 3C). The corresponding RNAs containing HPV-16del sequences both failed to give detectable cleavage in 3' dATP reactions, although some end polyadenylation of precursor RNA was detected by using ATP. These data indicate that the negative regulatory element does not alter the ability to process RNA in vitro.

Our studies both in vitro and in vivo indicate that by using HeLa cells, LP2 functions efficiently as a late HPV-16 mRNA 3' processing site. This suggests that the restriction in late-gene expression, observed in HeLa cells and other nondifferentiated epithelial cells, is unlikely to be due to the inability to process at a late poly(A) site. In contrast, LP1 functions inefficiently due to a poorly functional downstream GU-rich element; the LP1 signal does not function, perhaps for the same reason as LP1. This highlights the importance of the downstream GU-rich element in the functioning of HPV-16 late poly(A) sites. The GU-rich element of LP1 differs from the YGUGUUYY consensus sequence described by McLauchlan et al. (16) at positions six and seven; replacement of the last three nucleotides of this element with those of the LP2 GU-rich element converts LP1 to an efficient processing site. A similar result was obtained by Ryner et al. (23), who replaced the whole downstream sequences from a non-functional poly(A) site located within the coding region of the simian virus 40 large T antigen with those from the simian virus 40 late poly(A) site, thus generating a functional poly(A) site. McDevitt et al. (15) mutated the GU-rich element of the simian virus 40 early poly(A) site and identified certain positions crucial for efficient functioning in vivo; an A residue, also present in the LP1 GU-rich sequence (UGUGUAAC), was particularly unfavorable.

The reason for HPV-16 having two apparently nonfunctional poly(A) signals downstream of the late genes is unclear. One interesting possibility relates to the differential use of mRNA 3' processing signals in particular cell types. Differentiated keratinocytes are the only cells known to express papillomavirus late genes, and these cells could utilize 3' processing sites which are nonfunctional in other cell types. Since alteration of three nucleotides in the downstream GU-rich element greatly increases the efficiency of LP1, this suggests that any cell type specificity may reside in this 3' processing element, which does show sequence variability, rather than in the relatively invariant AAUAAA sequence. In some other systems in which alternative polyadenylation sites are utilized, alternative splicing pathways also are observed (1, 7, 13).

Our transfection studies indicate the presence of a negative regulatory element located immediately upstream of the late mRNA polyadenylation signals. In vitro polyadenylation analysis indicates that this element does not alter mRNA 3' processing. Studies in which an in vitro mRNA decay assay was used indicate that the negative element probably acts as an mRNA instability sequence (I. M. Kennedy, J. K. Haddow, and J. B. Clements, unpublished data). The high AU content and position of the negative element within the 3' untranslated region are consistent with instability elements found in other mRNAs (5, 10, 11, 20, 27).

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# Efficient Expression of the Human Papillomavirus Type 16 L1 Protein in Epithelial Cells by Using Rev and the Rev-Responsive Element of Human Immunodeficiency Virus or the *cis*-Acting Transactivation Element of Simian Retrovirus Type 1

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**Production of the human papillomavirus (HPV) late gene products L1 and L2 is limited to terminally differentiated keratinocytes. Here, we demonstrate that mRNA encoding the HPV-16 L1 capsid protein contains *cis*-acting RNA elements that inhibit expression at the posttranscriptional level. While cytoplasmic L1 mRNA is detectable in transfected HeLa cells, L1 protein is not produced. We have identified at least one major inhibitory element that is located within the L1 open reading frame, whereas another negative element had been reported to lie in the 3′-untranslated region of L1. The presence of these elements may explain the lack of HPV late gene expression in undifferentiated epithelial cells. Efficient production of HPV-16 L1 could be achieved with posttranscriptional regulatory elements of human immunodeficiency virus type 1 or simian retrovirus type 1. L1 protein was expressed in the presence of human immunodeficiency virus type 1 Rev from hybrid mRNAs containing the RNA binding site for Rev (Rev-responsive element). In addition, we have achieved efficient expression of L1 from hybrid mRNAs containing a *cis*-acting transactivation element from simian retrovirus type 1. Our data show that HPV-16 L1 protein production is regulated posttranscriptionally. This regulated expression may allow virus production in terminally differentiated epithelial cells and is probably a conserved and important mechanism for HPV expression.**

Human papillomaviruses (HPVs) are nonenveloped, double-stranded circular DNA viruses, which infect human epithelia and mucosa in a tissue-specific manner (41). The HPV genome is approximately 8,000 bp and consists of early and late genes as well as a noncoding region (NCR) (Fig. 1). Early genes encode proteins involved in viral replication, transformation, and transcriptional regulation, while late genes encode the viral capsid proteins L1 and L2 (64). The NCR contains *cis*-acting transcriptional and replicative elements in addition to elements that respond to keratinocyte-specific factors. The most frequently found cancer-associated HPV type is HPV-16, which is present in approximately 60% of cervical cancers (77). At present, HPVs are linked to up to 10% of the worldwide cancer burden, mainly because of their involvement in anogenital cancers (77). Cervical carcinoma biopsies isolated from the majority of cervical cancer patients contain HPV DNA. Because of the clinical importance and the risk of malignant progression of lesions induced by HPV-16, a better understanding of the molecular mechanisms that regulate the life cycle of HPV-16 is of particular importance.

The mechanisms which control HPV late gene expression are still not fully elucidated. Studies of the interaction of HPV with host cells and on the control of HPV late gene expression are hampered by the lack of an in vitro cell culture system for efficient propagation of virus. Analyses of basal cells in HPV-infected squamous epithelium have shown that HPV DNA is present at low copy numbers in these cells and that only early HPV genes are expressed (55). Concurrent with cell differentiation and migration of cells to suprabasal layers, HPV DNA copy number increases and expression of late genes is activated. In the differentiated cells, the HPV late proteins L1 and L2 are produced and virions are detected. The intimate association between HPV late gene expression and epithelial cell differentiation is likely to be regulated by *cis*-acting sequences on the viral genome or viral mRNAs and by transacting viral or cellular factors produced in infected cells. Studies of these regulatory mechanisms may provide valuable information for development of an efficient in vitro cell culture system for HPVs.

In situ hybridization studies of sections of HPV-33-infected tissue from a cervical condyloma accuminata (7) or from HPV-11-infected human xenografts in nude mice (71) revealed that L1 and L2 mRNAs produced in the middle layers of the infected epithelium were restricted to the nucleus, while high levels of L1 and L2 mRNAs were present in the cytoplasm in the superficial layers with differentiated cells. These observations suggested to us that expression of HPV late genes could be under posttranscriptional control. Expression of late genes of several retroviruses, such as human immunodeficiency virus type 1 (HIV-1), simian retrovirus type 1 (SRV-1), and Mason-Pfizer monkey virus, is posttranscriptionally regulated and has been well studied (9, 16, 17, 26, 33, 59, 61, 73, 76). Production

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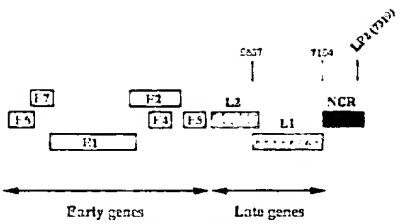


FIG. 1. Schematic representation of the genomic organization of HPV-16. Open boxes represent ORFs coding for early proteins E1 to E7, stippled boxes indicate ORFs coding for late proteins L1 and L2, and the solid box indicates the NCR. The nucleotide positions of the L1 translational start and stop codons and the major late poly(A) signal LP2 are indicated (43, 60). Numbers refer to nucleotide positions in the HPV-16 genomic clone pHPV16 (68).

of the retrovirus structural proteins Gag, Pol, and Env is regulated by positive and negative *cis*- and *trans*-acting factors. Here, we have used positive regulatory elements from HIV-1 and SRV-1 to investigate if the presence of these elements on HPV-16 late mRNAs could induce production of L1 capsid protein in human epithelial cells.

HIV-1, the etiologic agent of AIDS, is the prototype of the complex retroviruses (16, 26, 59). These viruses are characterized by the presence on their genomes of several short open reading frames (ORFs) coding for regulatory and accessory proteins. Two of these proteins, Tat and Rev, are regulators of gene expression and are essential for viral replication (16, 17, 26, 33, 59, 61, 73). Tat is a transcriptional transactivator, while Rev acts posttranscriptionally to induce expression of HIV-1 late genes. The Rev protein binds to an RNA sequence termed the Rev-responsive element (RRE) (1, 3, 4, 15, 20, 21, 24, 34, 37–40, 47, 53, 58, 70) that is present on late viral mRNAs coding for the structural proteins Gag, Pol, and Env. Rev overcomes the effect of inhibitory RNA sequences present on these mRNAs (10, 14, 51, 57, 62, 65, 67) and acts by promoting nuclear export of RRE-containing mRNAs (24, 25, 35, 53) and by increasing their stability (25, 67) and translatability (2, 18, 48). Therefore, Rev acts as a switch between early and late phases of viral gene expression in the HIV-1 life cycle.

Mason-Pfizer monkey virus and SRV-1 are type D retroviruses causing persistent infections in macaque monkeys (30). It has recently been shown that a *cis*-acting transactivation element (CTE) present on Mason-Pfizer monkey virus and SRV-1 mRNAs can substitute for the Rev-RRE regulatory system (9, 76), suggesting that factors similar in function to HIV-1 Rev are present in eucaryotic cells.

In the present study, we show that the HPV-16 L1 coding region contains inhibitory sequences that act posttranscriptionally to repress production of L1 capsid protein in the human epithelial cell line HeLa. Production of HPV-16 L1 protein could be induced from hybrid mRNAs containing the HIV-1 RRE in the presence of the HIV-1 Rev protein. Similarly, hybrid mRNAs containing the SRV-1 CTE expressed L1 protein efficiently. We also show that inhibition exerted by a previously identified negative element in the HPV-16 late 3′ untranslated region (UTR) [44] could be overcome by HIV-1 Rev and RRE or the SRV-1 CTE. Taken together, these results indicate that expression of HPV-16 late genes in nonkeratinized epithelial cells is likely to be regulated by the interaction of cellular factors with sequences present on viral late mRNAs. The results presented here favor a model for activation of HPV-16 late gene expression in infected squamous epithelium undergoing differentiation, which predicts either upregulation of positive factors antagonizing the negative elements or downregulation or inactivation of a cellular factor(s) presumably interacting with viral inhibitory sequences. This would allow HPV-16 late gene expression and production of virions.

## MATERIALS AND METHODS

Plasmid constructions. The complete HPV-16 L1 gene was PCR amplified from a previously described molecularly cloned HPV-16 genome (68) and was inserted into *Eco*RV-digested pBluescript, generating pT7-16L1. The HPV-16 L1 gene was transferred as a *Bss*HII-*Asp*718 fragment into *Bss*HII-*Asp*718-digested pNL17R (67), generating p16L1. p16L1RRE was generated by insertion of a 330-bp RRE-containing fragment (70) into *Asp*718-digested, calf intestinal alkaline phosphatase (CIAP)-treated p16L1. To generate p16L1CTE, the SRV-1 CTE-containing sequence was first PCR amplified from plasmid pS12 (76) with the oligonucleotides 12745 (5′-GCATCAACGGGTGTCGACGGATCCAGAC CACCTCCCCTGCGAG-3′) and 13827 (5′-GCATCAGCGGCCGCCTCGAG TCTAGACAAATCCCTCGGAAGCTGCG-3′) and cloned into *Eco*RV-digested pBluescript. Restriction sites introduced in oligonucleotides are underlined. The SRV-1 CTE was excised with *Xho*I, treated with a large fragment of Klenow DNA polymerase, and inserted into p16L1 digested with *Asp*718 and treated with Klenow and CIAP. These cloning steps resulted in plasmid p16L1CTE. To generate p16L1UTRRE, p16L1 was digested with *Eco*RI, treated with CIAP, and ligated to an *Eco*RI fragment, containing HPV-16 sequences from nucleotide (nt) 6818 to nt 7453 and the HIV-1 RRE inserted at position 7282.

To construct pNLCATW, the chloramphenicol acetyltransferase (CAT) gene was PCR amplified with the oligonucleotides CATS (5′-GCTAAGGAAGCTA AAATGGAG-3′) and CATA (5′-CTATTAGGCCCCGCCCTGCCACTC-3′) and subsequently cloned blunt ended into pNL17R (67), digested with *Sal*I and *Asp*718, and treated with CIAP and Klenow DNA polymerase. This resulted in plasmid pNLCATW. pNLCATW was digested with *Asp*718, followed by CIAP treatment and filling in of the 5′ overhang, and was used as a vector in the cloning steps described below. To construct CAT expression plasmids containing the complete HPV-16 L1 gene, the L1 coding sequence was PCR amplified and inserted into *Asp*718-digested pNLCATW. This generated pCATL1S and pCATL1A, containing the L1 gene in sense and antisense orientations, respectively. To generate derivatives of pCATL1S containing deletions in the L1 gene, various portions of the HPV-16 L1 gene were excised from pT7-16L1 (described above) with *Bss*HII in combination with *Eco*NI, *Pst*I, *Bst*XI, and *Bam*HI, filled in or trimmed, and cloned into *Asp*718-restricted pNLCATW, resulting in pCATL1ΔE, pCATL1ΔP, pCATL1ΔB, and pCATL1ΔBS, respectively. pCATL1SS was constructed by direct cloning of the *Ssp*I fragment (nt 5813 to 6551) of the L1 gene into *Asp*718-digested pNLCATW. pCAT-209 and pCAT-347 were generated by insertion of PCR fragments of the HPV-16 L1 gene, amplified with the oligonucleotides L1START and 16A (5′-CGGATCCGTAT TGTAATCCTGATACTTTAGGAAC-3′) or 16B (5′-CGGATCCGCCCACAC CTAATGGCTGACCACG-3′), respectively, into *Asp*718-digested pNLCATW. To generate derivatives of pCATL1S containing deletions in the 5′ end of the L1 gene, portions of HPV-16 L1 were PCR amplified with oligonucleotide L1STOP in combination with oligonucleotide 16-711 (5′-TTGGATCCGGTGTCAGA ACCATATGGCGACAGC-3′) or 16C (5′-CGGATCCCACAATAATGGCAT TTGTTGGGG-3′) and cloned into *Asp*718-digested pNLCATW, resulting in pCAT-804 and pCAT-565, respectively.

To construct pCH16pA, an *Eco*RI fragment containing the HPV-16 late 3′ UTR (nt 6818 to 7453) and late poly(A) signal was cloned into *Eco*RI-digested, CIAP-treated pBluescript. Uracil-containing single-stranded DNA was prepared as described previously (50) and used as the substrate for site-specific, oligonucleotide-directed mutagenesis to introduce a unique *Hpa*I site at nt 7282. The *Eco*RI fragment containing the mutated HPV-16 sequence was inserted into *Eco*RI-digested, CIAP-treated pCM1234, which contains a mutated HIV-1 $p17^{gag}$ gene, p17M1234 (65). This resulted in plasmid pCH16pA, in which the HPV-16 3′ UTR is located immediately downstream of p17M1234. p17M1234 contains multiple point mutations which altered the RNA sequence but maintained the original primary protein sequence of $p17^{gag}$ (65, 67). This resulted in high $p17^{gag}$ production independently of Rev (65, 67). p17M1234 is used as a reporter gene and is under control of the human cytomegalovirus (CMV) immediate early promoter (nt −671 to +74) (8). The HPV-16 late poly(A) signal LP2, located at nt 7319, has been shown to be efficiently utilized both in vitro and in vivo (43, 60) (Fig. 1 and 7). pCH16ΔpA was generated by digestion of pCH16pA with *Eco*NI (nt 6858) and *Hpa*I (nt 7282) and filling in of the 5′ overhang, followed by religation. pCH16RRE was generated by insertion of a 330-bp fragment spanning the HIV-1 RRE (70) into *Hpa*I-digested, CIAP-treated pCH16pA. To construct pCH16CS and pCH16CA, the SRV-1 CTE was PCR amplified from plasmid pS12 with oligonucleotides 12745 and 13827 (76) and subsequently cloned into *Hpa*I-digested, CIAP-treated pCH16pA, generating plasmids pCH16CS and pCH16CA, which contain the SRV-1 CTE in the sense and antisense orientations, respectively. pCM1234pA was constructed by insertion into an *Eco*RI-digested, CIAP-treated pCM1234 plasmid of an *Eco*RI

the present construct, the simian virus 40 (SV40) early poly(A) signal. To construct pT7-16pA and pT7-16ΔpA, sequences containing p17M1234 together with the HPV-16 3′ UTR or its truncated form were excised with *Sal*I and *Eco*RI from pCH16pA and pCH16ΔpA and cloned into pBluescript, resulting in plasmids pT7-16pA and pT7-16ΔpA, respectively.

Plasmid pCS1X was constructed by cloning of a DNA fragment (nt 7019 to 7525, numbers refer to the prototype HPV-1 sequence [19]) containing the HPV-1 late poly(A) signal into pHCMVSEAP (5) digested with *Hin*dIII and *Eco*RI. These cloning steps replaced the insulin poly(A)-containing sequence with the HPV-1 sequence. The Rev-producing plasmids pNL1.4A.7 (65) and pL-Rev (54), the SRV-1 CTE-containing plasmid pS12(*Bam*HI-*Xho*I) (76), and the HIV-1 *gag* plasmid pNL-*gag*-*pol*330 (70) have been described before.

**Cells and transfections.** HeLa cells or HLtat cells (65) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed as follows: $0.3 \times 10^5$ cells were plated onto 6-cm culture dishes 1 day before transfection. Transfections were carried out according to the calcium phosphate coprecipitation technique (32). The amount of plasmid used for transfection was adjusted with pBluescript DNA to 15 μg in 0.5 ml of precipitate for 60-mm-diameter dishes or 30 μg in 1 ml of precipitate for 100-mm-diameter dishes. Transfected cells were washed twice with phosphate-buffered saline (PBS) 6 h posttransfection and refed with fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and cultured overnight. Cells were harvested 24 h posttransfection. All plasmid DNAs used for transfection were purified on Qiagen columns (Diagen). One microgram of pCMVCAT, producing CAT enzyme, or pCS1X, producing secreted placental alkaline phosphatase (SEAP), was included as an internal control for transfection efficiency. Radioactive CAT assays were carried out by the method described by Gorman et al. (31). Production of CAT protein was quantitated with a CAT antigen capture enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim GmbH). To quantitate SEAP produced from the transfected cells, medium was collected and subjected to centrifugation at 13,000 × *g* for 2 min. The supernatants were incubated at 65°C for 15 min and then cooled to room temperature and incubated with alkaline phosphatase reagent (Sigma Diagnostics) for kinetic determination of alkaline phosphatase activity according to the manufacturer's recommendation.

Cells transfected with plasmid pT7-16pA, pT7-16ΔpA, or pT7-16L1 were first infected with recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase (27, 28). One hour postinfection, cells were transfected by the calcium phosphate precipitation technique.

**Western blot (immunoblot) analysis.** Cells were lysed in 500 μl of lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl [pH 7.5]), and lysates were cleared of insoluble material by centrifugation at 13,000 × *g* for 15 min. Forty-microliter aliquots of cell extracts were loaded on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes by electroblotting in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) at constant current (250 mA) for 12 to 16 h. Membranes were blocked for 1 h at 37°C with 10% nonfat milk in PBS containing 0.3% Tween 20 (PBS-T). After three washings in PBS-T buffer, the blots were incubated for 1 h at 37°C with serum. To detect HIV-1 $p17^{gag}$ or HPV-16 L1, HIV-1-positive patient serum or rabbit anti-HPV-16 L1 peptide serum (22) was used as the primary antibody, respectively, followed by incubation with horseradish peroxidase-conjugated anti-human or anti-rabbit immunoglobulin. Specific proteins were visualized with the enhanced chemiluminescence detection system (Amersham).

**Radioimmunoprecipitation assay.** Transfected cells were washed twice with prewarmed PBS 24 h posttransfection and then washed twice with methionine-free 1× Eagle's minimum essential medium. The cells were starved for 30 min at 37°C in 1.0 ml of Met-free medium containing 0.5% FCS, followed by metabolic labeling for 1 h with 200 μCi of [$^{35}$S]Met (Amersham). The cells were washed with cold PBS and harvested in 600 μl of ice-cold 0.5× radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-Cl [pH 7.4], 75 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS). After three freeze-thawings, cell debris was removed by centrifugation at 15,000 × *g* at 4°C for 15 min. The supernatants were mixed with preimmune rabbit serum and incubated at 4°C for 1 h, followed by addition of protein A-Sepharose (Pharmacia) suspension and incubation at 4°C for 30 min. Samples were centrifuged, and supernatants from each sample were collected and aliquoted into two Eppendorf tubes, to which either normal rabbit serum or rabbit anti-HPV-16 L1 peptide serum (22) was added. Incubation was kept at 4°C for 12 to 16 h with gentle rocking. Protein A-Sepharose was added, and samples were incubated for 3 h at 4°C. After centrifugation, the Sepharose beads were washed three times in 1× RIPA buffer and resuspended in 5× SDS-loading buffer (625 mM Tris [pH 6.8], 6.25% SDS, 50% glycerol, 20% 2-mercaptoethanol), boiled, and loaded on SDS–10% polyacrylamide gels under reducing conditions. Immunoprecipitated proteins were visualized by autoradiography (Kodak XAR film) at −70°C.

**Cell fractionation and extraction of poly(A)$^+$ mRNA.** Nuclear and cytoplasmic poly(A)$^+$ mRNA was isolated with Dynabeads oligo$(dT)_{25}$ (Dynal A.S.). Twenty-four hours posttransfection, cells from 100-mm-diameter dishes were washed twice with ice-cold PBS, followed by lysis in 500 μl of lysis buffer D (10 mM Tris-HCl [pH 7.5], 0.14 M NaCl, 5 mM KCl, 1% Nonidet P-40). Nuclear and cytoplasmic fractions were separated by low-speed centrifugation at 8,000 × *g* for 2 min. To extract cytoplasmic mRNA, supernatants were mixed with an equal volume of 2× binding buffer (20 mM Tris-HCl [pH 7.5], 1.0 M LiCl, 2 mM EDTA, 0.5% SDS) containing 5 μl per Dynabeads oligo$(dT)_{25}$. Hybridization was carried out at room temperature for 3 to 5 min, followed by collection of Dynabeads oligo$(dT)_{25}$ three times with 500 μl of washing buffer (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 2 mM EDTA). The poly(A)$^+$ mRNAs were eluted with 150 μl of elution buffer (2 mM EDTA [pH 7.5]) at 65°C for 5 min, and stored at −70°C until use. Nuclear fractions were washed once with lysis buffer D, and the pellets were resuspended in lysis buffer D without Nonidet P-40. An equal volume of lysis buffer D containing 1% SDS was added, and the samples were incubated on ice for 10 min, followed by freezing on dry ice. The samples were thawed at room temperature and centrifuged for 2 min at 8,000 × *g*. Supernatants were used for extraction of nuclear poly(A)$^+$ mRNA with Dynabeads oligo$(dT)_{25}$ as described above.

**RT-PCR.** Twenty microliters of poly(A)$^+$ mRNA or dilutions thereof were reverse transcribed at 42°C for 1 h in a total volume of 30 μl, containing 4 U of avian myeloblastosis virus reverse transcriptase (RT, Promega), 19 U of RNA guard (Pharmacia), 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 50 μg of random hexamer oligonucleotides per ml (Pharmacia). A reaction without RT was performed in parallel and served as a control for the absence of plasmid DNA contamination. Five microliters of the cDNA product was PCR amplified in a 100-μl reaction volume with oligonucleotides 4704 (5′-GCCTGTTAGAAACATCAGAAGGC-3′) and 5912 (5′-CTAGTAATTTTGGCTGACCTG-3′) detecting cDNA of $p17^{gag}$ or $p55^{gag}$ mRNA; oligonucleotides 16G (5′-CGATATCCCAGATACAC AGCGGCTGGTTTG-3′) and 16M (5′-CGGATCCCACAATTGTGTTTGT TTGTAATCC-3′), detecting cDNA of L1 mRNA; ACTINS (5′-TGAGCTGCG TGTGGCTCC-3′) and ACTINA (5′-GGCATGGGGGAGGGCATACC-3′), specifically amplifying cDNA of spliced actin mRNA; or ACTINS-1 (5′-CCAGT GGCTTCCCCAGTG-3′) and ACTINA, detecting cDNA of unspliced actin mRNA. To detect cDNA of CAT mRNA produced from the CAT expression plasmid used as an internal control for transfection efficiency, oligonucleotides CATS-2 (5′-CGTCTCAGCCAATCCCTGGGTG-3′) and CATA (5′-CTATT AGGCCCCGCCCTGCCACTC-3′) were used. Oligonucleotides were purchased from Scandinavian Gene Synthesis.

A 250-bp $p17^{gag}$ fragment was PCR amplified by oligonucleotides 4704 and 5912, a 199-bp product was amplified by 16G and 16M, a 247-bp product was amplified by ACTINS and ACTINA, a 203-bp product was amplified by ACTINS-1 and ACTINA, and a 229-bp fragment was amplified by CATS-2 and CATA. Oligonucleotides 5912, 16 M, ACTINA, and CATA were end labeled with [$\gamma$-$^{32}$P]ATP prior to use. PCR amplification was performed in a total volume of 100 μl with 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) for 20 to 25 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s with a final extension at 72°C for 10 min. Ten microliters of each RT-PCR mixture was analyzed by electrophoresis on 6% polyacrylamide gels with or without urea. The gels were dried and exposed to X-ray film at −70°C.

**Northern RNA blotting.** At day 2 posttransfection, cytoplasmic RNA was extracted from duplicate plates (18) and Northern blots were prepared as described previously (34). The L1 mRNAs were detected with a probe spanning nt 8304 to 9008 of the HIV-1 molecular clone HXB2. Subsequently, the same blots were probed with a 2-kb *Hin*dIII fragment of β2000 plasmid (12) to detect β-actin mRNAs.

## RESULTS

**Efficient production of the HPV-16 L1 protein with a vaccinia virus-T7 RNA polymerase-based expression system.** The HPV-16 genome can be divided into early and late regions (Fig. 1). Expression of HPV-16 late genes (L1 and L2) is restricted to terminally differentiated epithelial cells in genital mucosa. To study expression of HPV-16 L1 protein in nonkeratinized cells, we introduced HPV-16 L1 gene expression vectors into HeLa cells, a human epithelial cell line that resembles cells in the basal layer of stratified epithelium. Two types of expression systems were employed: a vaccinia virus-T7 RNA polymerase-based expression system (27, 28), which allows transcription of the gene of interest in the cytoplasm, and eucaryotic expression plasmids containing promoters which depend on nuclear transcription factors. Since it has been demonstrated that an inhibitory element is present in the HPV-16 late 3′ UTR (44), the HPV-16 late 3′ UTR was excluded from the plasmids shown in Fig. 2A.

To circumvent nuclear regulatory mechanisms of gene expression, the vaccinia virus-T7 RNA polymerase-based expression system (27, 28) was utilized. The HPV-16 L1 gene was cloned downstream of the bacteriophage T7 promoter, generating plasmid pT716L1 (Fig. 2A). Infection of HeLa cells with

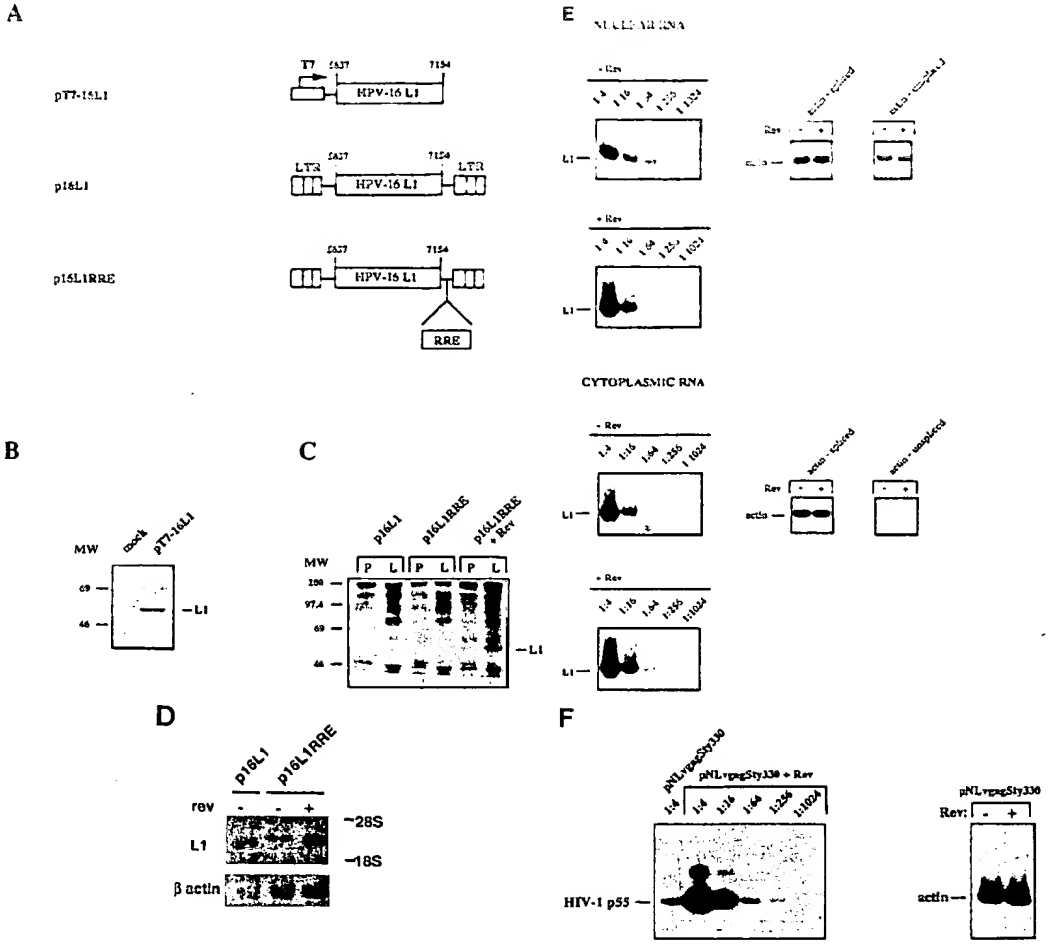


FIG. 2. HIV-1 Rev and RRE activate production of HPV-16 L1 protein. (A) Schematic structures of the HPV-16 L1 expression plasmids. The small open box at the top represents the bacteriophage T7 promoter, and the bent arrow indicates the direction of transcription driven by the T7 promoter. Large open boxes indicate HPV-16 sequences or the HIV-1 RRE as indicated. Numbers indicate nucleotide positions on the genomic HPV-16 clone (68). Light stippled boxes represent the HIV-1 LTR. The names of the plasmids are indicated on the left. (B) Western blot analysis of the HPV-16 L1 protein. HeLa cells were first infected with recombinant vaccinia virus vTF7-3 (27, 28) and then were transfected with pT7-16L1. Cells were harvested 24 h posttransfection, and proteins were separated on SDS–12% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The L1 protein was detected with a rabbit anti-HPV-16 L1 peptide antiserum (22), followed by incubation with horseradish peroxidase-conjugated anti-human immunoglobulin G antiserum. The L1 protein was visualized with enhanced chemiluminescence detection reagents. The position of the L1 protein is indicated on the right. MW, molecular mass markers (in kilodaltons). (C) Radioimmunoprecipitation of HPV-16 L1 protein. HLtat cells were transfected with p16L1 or p16L1RRE in the absence or presence of Rev. Twenty-four hours posttransfection, cells were labeled with [$^{35}$S]methionine for 1 h. Immunoprecipitation was performed with either rabbit preimmune serum or rabbit anti-HPV-16 L1 peptide antiserum (22). Proteins were separated on SDS–12% polyacrylamide gels and visualized by autoradiography. P, rabbit preimmune serum; L, rabbit anti-HPV-16 L1 peptide antiserum. The position of the p55 L1 protein is indicated on the right. (D) Northern blot analysis of cytoplasmic RNA from HLtat cells, transiently transfected with the indicated plasmids in the presence or absence of an HIV-1 Rev expression plasmid, pLsRev. The positions of the rRNAs are indicated. (E) Analysis of subcellular distribution of HPV-16 L1 mRNAs. Nuclear and cytoplasmic poly(A)$^+$ mRNAs were isolated from cells transfected with p16L1 or from cells transfected with p16L1RRE in the presence of Rev and were subsequently reverse transcribed and PCR amplified with oligonucleotides end labeled with [$\gamma$-$^{32}$P]ATP. The PCR products were analyzed on 6% polyacrylamide gels, followed by autoradiography. The upper left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted nuclear poly(A)$^+$ mRNAs isolated from cells transfected with p16L1 in the absence of HIV-1 Rev or from cells transfected with p16L1RRE in the presence of HIV-1 Rev. The positions of the amplified DNA fragments are indicated on the left. The upper right panel shows PCR amplification of cDNA from spliced or unspliced actin mRNA in the nuclear fraction of transfected cells to control for cell fractionation. The number of cycles used for PCR amplification of cDNA was adjusted to detect cDNA in the linear range of the assay. The lower left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)$^+$ mRNA isolated from cells transfected with p16L1 in the absence of HIV-1 Rev or from cells transfected with p16L1RRE in the presence of HIV-1 Rev. The lower right panel shows PCR amplification of cDNA from spliced and unspliced actin mRNAs in the cytoplasmic fraction of transfected cells. (F) RT-PCR analysis of cytoplasmic poly(A)$^+$ mRNAs. Serial fourfold dilutions of mRNA samples from cells transfected with pNLvgagSty330 (70) in the absence or presence of Rev were performed, and cDNAs were generated from each dilution. PCR was performed with oligonucleotides specific for the HIV-1 p55$^{gag}$ mRNA produced by pNLvgagSty330 (left panel) or with oligonucleotides specific for spliced actin mRNA as a control for RNA levels (right panel). The positions of PCR products are indicated on the left.

the recombinant vaccinia virus vTF7-3 (27, 28), producing bacteriophage T7 RNA polymerase, is a prerequisite for expression of the L1 gene. The virally produced T7 RNA polymerase localizes to the cytoplasm and specifically recognizes the bacteriophage T7 promoter (27, 28). Since T7 RNA polymerase produced by vTF7-3 is a large protein lacking a nuclear localization signal, it does not enter the nucleus (23). Therefore, transcription directed by the T7 RNA polymerase takes place primarily in the cytoplasm. Figure 2B shows that transfection of pT7-16L1 into HeLa cells infected with recombinant vaccinia virus vTF7-3 gave rise to high levels of the HPV-16 L1 protein, as determined by Western blots with an HPV-16 L1-specific antipeptide antiserum (22).

**Lack of L1 protein production with eucaryotic expression vectors.** Expression of HPV-16 L1 protein was also examined with plasmids in which the L1 gene was under the control of a promoter that requires nuclear localization for function. We constructed plasmid p16L1, in which the HPV-16 L1 gene coding sequence is under the control of the HIV-1 long terminal repeat (LTR) promoter (Fig. 2A). Plasmid p16L1 was transfected into HLtat cells (66), a HeLa-derived cell line constitutively producing the HIV-1 Tat protein required for transcription from the HIV-1 LTR. We could not detect L1 protein expression in HLtat cells transfected with p16L1 by radioimmunoprecipitation with a rabbit anti-HPV-16 L1 peptide antiserum (Fig. 2C) (22). In conclusion, high levels of HPV-16 L1 protein were produced with the vaccinia virus-T7 RNA polymerase-based expression system, while L1 protein was undetectable in cells transfected with p16L1, which depends on nuclear factors. These observations suggested that inhibition of HPV-16 L1 gene expression may involve nuclear events.

**HIV-1 Rev and RRE activate production of HPV-16 L1 protein.** HIV-1 produces the posttranscriptional transactivator protein Rev, which binds to the RRE RNA site and is required for production of the HIV-1 structural proteins Gag, Pol, and Env. We wished to investigate whether the HIV-1 Rev protein could activate expression of HPV-16 L1. Therefore, a 330-nt fragment encompassing HIV-1 RRE was first introduced into plasmid p16L1, resulting in p16L1RRE (Fig. 2A). This plasmid was transfected into HLtat cells in the absence or presence of HIV-1 Rev-producing plasmid pNL1.4A.7 (66). Results of radioimmunoprecipitations showed that L1 protein was detected only in the presence of HIV-1 Rev and RRE (Fig. 2C). A CAT-expressing plasmid, pCMVCAT, was included as an internal control in each transfection experiment. The amount of CAT protein in each sample did not vary more than twofold among these transfections (data not shown). We concluded that HIV-1 Rev and RRE could activate expression of HPV-16 L1 in human HLtat cells of epithelial origin.

**L1 mRNAs accumulate in the cytoplasm but are not efficiently translated.** Since previous studies had established that HIV-1 Rev acts at least partially by promoting nuclear export of HIV-1 mRNAs (24, 25, 35, 53), thereby affecting subcellular distribution of RRE-containing mRNAs, we analyzed nuclear and cytoplasmic L1 mRNA levels in the absence or presence of HIV-1 Rev and RRE. Northern blot analysis revealed that intact L1 mRNA was readily detectable in the cytoplasmic fraction (Fig. 2D). The presence of Rev resulted in a small increase in mRNA produced from p16L1RRE. To quantitate the levels of mRNA, we subjected poly(A)$^+$ mRNA to RT-PCR analysis. Nuclear and cytoplasmic poly(A)$^+$ mRNAs were extracted from HLtat cells transfected with p16L1 in the absence of Rev or from HLtat cells transfected with p16L1RRE in the presence of Rev. Serial fourfold dilutions of the mRNA samples were subjected to random primed cDNA synthesis followed by PCR with oligonucleotides specific for the HPV-16 L1 sequence. Figure 2E revealed that high levels of HPV-16 L1 mRNAs were present in the cytoplasm in the absence of Rev and RRE. An increase in cytoplasmic L1 mRNA levels estimated to be less than fourfold was observed in the presence of Rev (Fig. 2E). Similarly, L1 mRNA levels in the nucleus were also slightly increased in the presence of Rev (Fig. 2E). Levels of CAT protein produced from the internal control plasmid pCMVCAT were analyzed in the same transfections and varied less than twofold between the transfections (data not shown).

It could be argued that HPV-16 L1 mRNAs trapped in the nucleus leak out into the cytoplasm during cell fractionation. To control for the fractionation technique, we analyzed unspliced and spliced actin mRNAs by RT-PCR. While spliced actin mRNA was found in both nuclear and cytoplasmic fractions, unspliced actin mRNA was primarily found in the nuclear fraction (Fig. 2E), demonstrating that nuclei remained intact during fractionation. In parallel transfections, we analyzed cytoplasmic HIV-1 *gag* mRNA levels produced from pNLvgagSty330 (25, 70) in the absence or presence of Rev. The results showed a big increase in cytoplasmic $p55^{gag}$ mRNA levels in the presence of Rev, while similar levels of cytoplasmic actin mRNA were detected (Fig. 2F). This is in agreement with previous studies that established that the majority of the HIV-1 *gag* mRNAs produced from this plasmid are retained in the nucleus in the absence of Rev (25). We concluded that inhibition of HPV-16 L1 production was not caused exclusively by entrapment of L1 mRNAs in the nucleus. Although Rev did increase the levels of L1 mRNAs in the cytoplasm, the increase in L1 protein levels appeared to be greater than that in L1 mRNA levels, suggesting that Rev acted by also increasing translation of the HPV-16 L1 mRNAs.

It has been shown that the HPV-16 late 3′ UTR contains sequences that inhibit gene expression (44). Since such sequences are normally present on L1 mRNAs, we wished to determine whether Rev and RRE could activate L1 production from an L1 mRNA also containing the HPV-16 late 3′ UTR. Therefore, RRE was inserted into a plasmid containing the entire HPV-16 L1 gene and 3′ UTR sequence, resulting in plasmid p16L1UTRRE (Fig. 3A). L1 production from this plasmid in the absence or presence of Rev was assessed by radioimmunoprecipitation. Figure 3B shows that L1 protein was produced from p16L1UTRRE only in the presence of Rev. The levels of L1 protein were similar between p16L1UTRRE and p16L1RRE in the presence of Rev (Fig. 3B). These results demonstrated that Rev could also activate L1 expression when the previously described inhibitory sequences in the HPV-16 late 3′ UTR (44) were present on the mRNAs.

**The SRV-1 CTE acts in *cis* to induce HPV-16 L1 expression.** It was recently reported that the 3′ UTR of Mason-Pfizer monkey virus and SRV-1 contains an RNA sequence, CTE (9, 76), which is able to substitute for HIV-1 Rev and RRE and render HIV-1 structural gene expression Rev independent. It is thought that cellular factors analogous to Rev interact with CTE. It was therefore of interest to test whether CTE could activate expression of HPV-16 L1 protein. The SRV-1 CTE was inserted downstream of the L1 gene in plasmid p16L1, generating plasmid p16L1CTE (Fig. 3A). HLtat cells were transfected with p16L1CTE and metabolically labeled with [$^{35}$S]methionine. Radioimmunoprecipitation showed that high levels of L1 protein were produced from p16L1CTE, demonstrating that the presence of the SRV-1 CTE in a sense orientation induced production of L1 protein (Fig. 3B). Comparison of the HPV-16 L1 protein levels induced by Rev and RRE or

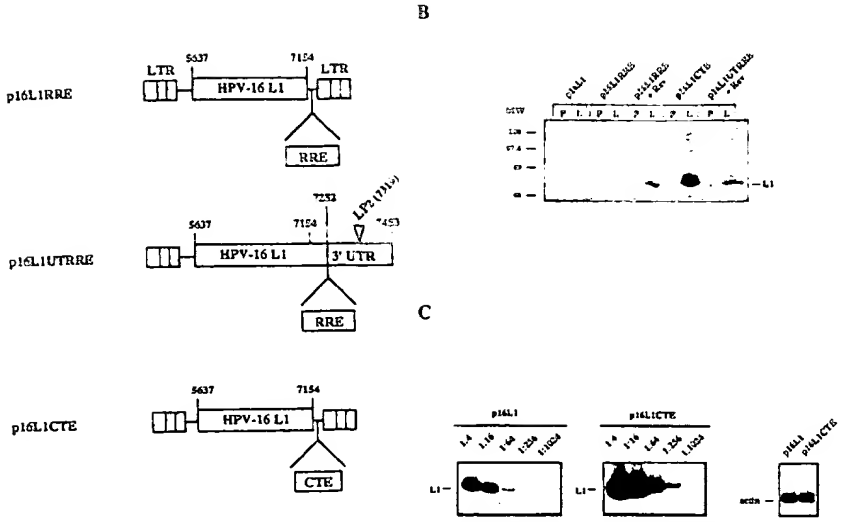


FIG. 3. The presence of HIV-1 Rev in *trans* and RRE in *cis* or the SRV-1 CTE element in *cis* activates expression of the HPV-16 L1 capsid protein. (A) Schematic structures of HPV-16 L1 expression plasmids. Lightly shaded boxes indicate HIV-1 LTRs. Open boxes indicate HPV-16 sequences (HIV-1 RRE or SRV-1 CTE) as indicated. The position of the translational stop codon at position 7154 is indicated. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). The small open triangle represents the major late poly(A) signal LP2 present in the HPV-16 3' UTR (43, 60). (B) Immunoprecipitation of the HPV-16 L1 protein. HLtat cells were transfected with the various plasmids shown in panel A. Cell lysates were subjected to immunoprecipitation with rabbit preimmune serum or rabbit anti-HPV-16 L1 peptide antiserum (22). Proteins were analyzed on SDS–12% polyacrylamide gels and visualized by autoradiography. The position of the HPV-16 L1 protein is indicated on the right. P, rabbit preimmune serum; L, rabbit anti-HPV-16 L1 peptide antiserum; MW, molecular mass marker (in kilodaltons). (C) SRV-1 CTE increases the HPV-16 L1 mRNA levels in the cytoplasm. HLtat cells were transfected with p16L1 or p16L1CTE. Cytoplasmic poly(A)$^+$ mRNAs isolated from transfected cells were reverse transcribed and subjected to PCR amplification with oligonucleotides specific for HPV-16 L1 mRNA. Oligonucleotides were end labeled with [$\gamma$-$^{32}$P]ATP prior to PCR. The amplified products were analyzed on 6% polyacrylamide gels and examined by autoradiography. The left panel shows PCR amplification of cDNAs synthesized from serially fourfold-diluted cytoplasmic poly(A)$^+$ mRNA isolated from cells transfected with p16L1 or p16L1CTE. The positions of the PCR products are indicated on the left. The right panel shows RT-PCR analysis of spliced actin mRNA used to control for the amount of mRNA used for RT-PCR.

by the CTE element revealed that L1 levels produced in the presence of CTE were significantly higher than those produced in the presence of Rev and RRE (Fig. 3B). This was consistently observed in three independent experiments. Therefore, in these cells, CTE was a better activator of L1 expression.

We next analyzed mRNA levels produced in the absence or presence of the CTE element in *cis*. poly(A)$^+$ mRNA was extracted from HLtat cells transfected with p16L1 or p16L1CTE. The mRNA samples were serially diluted and subjected to cDNA synthesis and PCR amplification. Figure 3C shows that the cytoplasmic poly(A)$^+$ mRNA levels produced from p16L1CTE were four- to eightfold higher than those produced from p16L1. The amounts of CAT produced from the internal control plasmid pCMVCAT varied less than twofold between the transfections (data not shown). Actin mRNA levels were similar in both RNA samples (Fig. 3C), demonstrating that equal amounts of poly(A)$^+$ mRNA were analyzed in RT-PCR. We concluded that the CTE element acted by increasing both cytoplasmic L1 mRNA levels and L1 protein levels similar to what was described above for Rev and RRE. The increase appeared to be greater at the protein level than at the mRNA level, indicating that CTE acts by increasing both cytoplasmic L1 mRNA levels and mRNA translation.

**Identification of *cis*-acting inhibitory sequences in the HPV-16 L1 coding region.** Since HPV-16 L1 protein was produced only in the presence of the CTE or Rev and RRE, these results suggested that either L1 mRNAs lack *cis*-acting RNA sequences required for efficient mRNA processing and utilization or that inhibitory sequences are present in the HPV-16 L1 coding sequence. To investigate if the L1 sequence contains an inhibitory element, we asked whether the presence of the L1 coding sequence downstream of the CAT reporter gene could inhibit CAT production. The entire L1 coding sequence was inserted in a sense or antisense orientation downstream of the CAT gene in plasmid pNLCATW (Fig. 4A), resulting in pCATL1S and pCATL1A, respectively (Fig. 4A). CAT activity or CAT protein levels in HLtat cells transfected with pCATL1S, pCATL1A, or pNLCATW were evaluated. Figure 4B shows that low levels of CAT activity were produced from pCATL1S, while pCATL1A and pNLCATW produced high levels of CAT, demonstrating that the L1 coding sequence inhibits CAT production in an orientation-dependent manner.

We also used a CAT ELISA specifically detecting CAT protein to quantitate the amount of CAT present in the cell extracts analyzed for Fig. 4B. The results revealed that plasmid pCATL1S produced 78-fold lower levels of CAT than did pNLCATW (Fig. 4C). The presence of the L1 gene in an antisense orientation, as in pCATL1A, only resulted in a two- to threefold reduction in CAT production (Fig. 4C). Similar results were obtained from three independent experiments. Plasmid pCS1X, producing SEAP, was included in these transfections and served as an internal control for transfection efficiency. SEAP production did not vary more than twofold between the different transfections (data not shown). The two- to threefold inhibitory effect on CAT production observed with the HPV-16 L1 sequence in the antisense orientation is be-

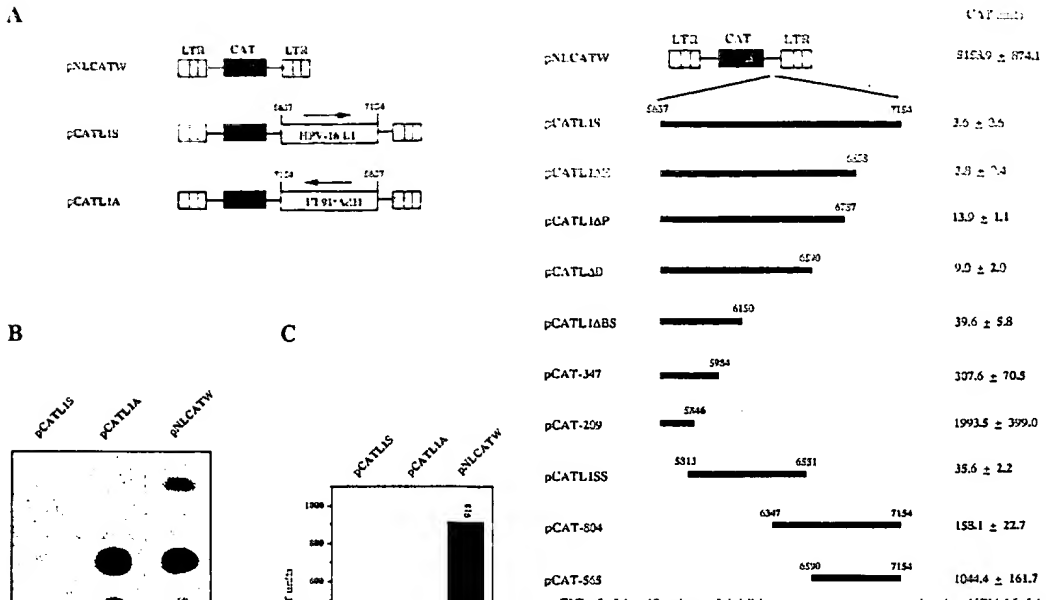


FIG. 4. HPV-16 inhibitory element inhibits CAT expression in an orientation-dependent manner. (A) Schematic structures of CAT expression plasmids. Lightly shaded boxes represent the HIV-1 LTRs. Darkly shaded boxes indicate the CAT gene. Open boxes represent HPV-16 L1 coding sequences. Numbers indicate nucleotide positions on the genomic HPV-16 clone (68). Arrows indicate orientation of the HPV-16 L1 gene in the plasmid. The names of the plasmids are indicated on the left. (B) The presence of the HPV-16 L1 gene in the sense orientation strongly inhibits CAT activity. HLtat cells transfected with pNLCATW, pCATL1S, and pCATL1A were harvested in lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl [pH 7.8]). CAT activity in each cell lysate was analyzed in triplicate by the method described by Gorman et al. (31). Results from a representative experiment are shown. (C) CAT protein levels from the same samples used for measuring CAT activities in Fig. 4B were determined by a CAT-capture ELISA kit (Boehringer Mannheim GmbH). CAT protein levels are displayed as CAT units.




FIG. 5. Identification of inhibitory sequences present in the HPV-16 L1 coding region. Lightly shaded boxes represent the HIV-1 LTRs. The darkly shaded box indicates the CAT gene. Solid bars represent the HPV-16 L1 coding sequence. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). The names of the plasmids are shown on the left. Triplicate transfections were performed. The difference in transfection efficiency among CAT expression plasmids was corrected by being normalized to SEAP produced from pCS1X, included as an internal control. Arithmetic means and standard deviations were calculated by standard methods and are shown on the right.

lieved to be unspecific and is too low to account for the inhibitory effect on L1 production. We concluded that the HPV-16 L1 coding sequence contained *cis*-acting inhibitory elements that acted in an orientation-dependent manner to inhibit gene expression.

**An inhibitory element located between nt 5813 and 6150 in the HPV-16 L1 coding sequence.** To map the inhibitory sequences, we generated a series of deletions in the 3′ end of the L1 coding sequence in pCATL1S (Fig. 5). Triplicate transfections were performed and CAT levels were determined with the CAT ELISA as described above. The CAT values were normalized to those of SEAP produced from plasmid pCS1X, included in each transfection as an internal control for transfection efficiency. Deletions in the 3′ end of L1 in pCATL1S resulted in a gradual increase in CAT levels. The L1 fragment present in pCATL1ΔBS substantially inhibited CAT expression, while higher levels of CAT were produced from pCAT-347 (Fig. 5). These experiments mapped the 3′ boundary of a negative element to sequences between nt 5984 and 6150. To investigate if inhibitory sequences were present in the 5′ end of the L1 gene, 5′ deletions were introduced in the L1 gene in plasmid pCATL1S. Deletion of sequences between nt 5637 and 6347 as in plasmid pCAT-804 resulted in increased levels of CAT protein. However, these levels were significantly lower than those produced from pNLCATW. Therefore, inhibitory sequences were present also between nt 6150 and 7154. Interestingly, plasmid pCAT-565 produced high levels of CAT, demonstrating that inhibitory sequences were entirely or partially deleted in this plasmid. Plasmid pCATL1SS, containing an L1 fragment spanning sequences between nt 5813 and 6551 near the 5′ end of the L1 coding sequence, produced low levels of CAT. These results indicated that sequences between nt 5637 and 5813 were not required for strong inhibition. These results suggest that one inhibitory element is located between nt 5813 and 6150 and that another is located downstream of nt 6150.

To investigate the effect of the L1 sequence on CAT mRNA levels, we analyzed cytoplasmic poly(A)$^+$ mRNA levels in HLtat cells transfected with pNLCATW or pCATL1SS (Fig. 6A). RT-PCR was performed on serially diluted RNA samples with oligonucleotides specifically detecting CAT mRNAs. The results showed that CAT mRNA levels generated from pCATL1SS were fourfold lower than those produced from pNLCATW (Fig. 6B). Levels of SEAP produced from pCS1X, included to serve as a control for transfection efficiency, varied

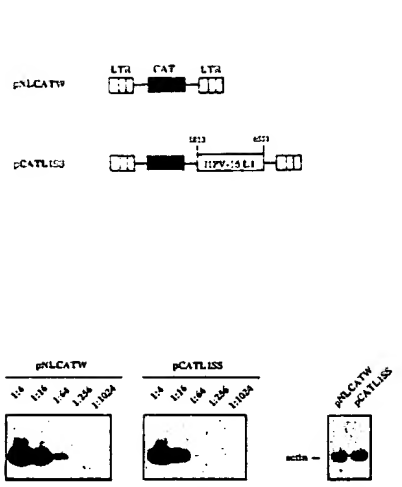


FIG. 6. HPV-16 L1 coding sequence (nt 5813 to 6551) is necessary and sufficient for inhibition. (A) Schematic structures of CAT expression plasmids. Lightly shaded boxes represent the HIV-1 LTRs. Darkly shaded boxes indicate the CAT gene. The open box represents the HPV-16 L1 coding sequence. Numbers indicate nucleotide positions on the genomic HPV-16 clone (68). Names of the plasmids are shown on the left. (B) Analysis of cytoplasmic poly(A)$^+$ mRNA levels from HLtat cells transfected with pNLCATW or pCATL1SS. The left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)$^+$ mRNAs isolated from cells transfected with pNLCATW or pCATL1SS. The positions of the amplified DNA fragments are indicated on the left. The oligonucleotides were end labeled with [$\gamma$-$^{32}$P]ATP prior to PCR. The PCR products were analyzed on 6% polyacrylamide gels, followed by autoradiography. The right panel shows PCR amplification of cDNA from spliced actin mRNA in the cytoplasmic fraction of transfected cells. The number of cycles for PCR amplification of cDNA was adjusted to detect the amplified products in the linear range of the assay.

less than twofold between transfections (data not shown). These results verified that the presence of inhibitory sequences in HPV-16 L1 decreased cytoplasmic poly(A)$^+$ mRNA levels. However, the difference in CAT protein production between pNLCATW and pCATL1SS was approximately 140-fold (Fig. 5). Therefore, the inhibitory effect was greater at the protein level than at the mRNA level. These results demonstrated that mRNAs containing inhibitory HPV-16 sequences were present in the cytoplasm but were not efficiently translated. We concluded that inhibitory L1 sequences acted by reducing both cytoplasmic poly(A)$^+$ mRNA levels and mRNA utilization.

**Inhibitory sequences in the HPV-16 late 3′ UTR act by reducing mRNA levels and mRNA utilization.** It has been reported that the HPV-16 late 3′ UTR contains inhibitory sequences that act in *cis* to reduce expression of a reporter gene in transient transfection experiments (44). To investigate whether these sequences were functionally similar to the negative element in the HPV-16 L1 coding sequence identified here, we inserted an HPV-16 sequence (nt 6818 to 7453), containing the HPV-16 late 3′ UTR and late poly(A) signal, downstream of a CMV promoter and an HIV-1 p17$^{gag}$ gene used here as a reporter gene (65) (see Materials and Methods). This resulted in plasmid pCH16pA (Fig. 7A). A deletion which removed the previously identified negative element was introduced into pCH16pA, resulting in pCH16ΔpA (Fig. 7A). Production of p17$^{gag}$ from cells transfected with pCH16pA or pCH16ΔpA was assessed by Western immunoblotting. Figure 7B showed that pCH16ΔpA produced high levels of p17$^{gag}$, while pCH16pA produced only low levels of p17$^{gag}$. The levels of p17$^{gag}$ produced from pCH16ΔpA were similar to those produced from pCM1234pA (Fig. 7B), in which the HPV-16 late poly(A) signal was replaced with the SV40 early poly(A) signal (Fig. 7A). Western blot analysis of serial 2-fold dilutions with extracts from cells transfected with pCH16ΔpA revealed that approximately 64-fold higher levels of p17$^{gag}$ were produced from pCH16ΔpA compared with those produced from pCH16pA (Fig. 7C). This is in agreement with previous studies of the inhibitory effect of this region of HPV-16 (44). Plasmid pCMVCAT was included as an internal control for transfection efficiency, and CAT levels did not vary more than twofold between the different transfections (data not shown).

To determine whether the 64-fold difference in p17$^{gag}$ production between pCH16ΔpA and pCH16pA was reflected at the mRNA level, we analyzed cytoplasmic poly(A)$^+$ mRNA produced from pCH16ΔpA and pCH16pA, respectively. The cytoplasmic poly(A)$^+$ mRNA samples were serially diluted and subjected to random hexamer oligonucleotide-primed reverse transcription followed by PCR amplification with oligonucleotides specific for p17$^{gag}$ mRNA or oligonucleotides specific for CAT mRNA produced from the internal control plasmid pCMVCAT. The RT-PCR results revealed that pCH16pA produced approximately fourfold lower levels of cytoplasmic poly(A)$^+$ mRNA than did pCH16ΔpA (Fig. 7D), while CAT mRNA levels did not vary substantially (Fig. 7D). Similar to the inhibitory sequences in the L1 coding region, the negative element in the HPV-16 late 3′ UTR acted in *cis* to decrease both cytoplasmic mRNA levels and mRNA utilization.

**HIV-1 Rev and RRE can overcome the effect of the inhibitory element in the HPV-16 late 3′ UTR.** Since the presence of HIV-1 Rev in *trans* and RRE in *cis* could overcome the effect of the inhibitory sequences in the L1 coding sequence, we next tested if Rev and RRE could also overcome the effect of the inhibitory element in the HPV-16 late 3′ UTR. RRE was therefore inserted into plasmid pCH16pA, generating pCH16RRE (Fig. 8A).[9] Analysis of p17$^{gag}$ production from this plasmid in the absence or presence of Rev revealed that Rev could overcome the negative effects of the inhibitory HPV-16 element (Fig. 8B). The p17$^{gag}$ levels produced from pCH16RRE in the presence of Rev were similar to those produced from pCM1234pA (Fig. 8B), demonstrating that HIV-1 Rev was able to efficiently restore levels of expression of p17$^{gag}$ protein. A quantitative estimate of p17$^{gag}$ protein levels produced in the absence or presence of Rev revealed that the presence of Rev increased p17$^{gag}$ protein levels by approximately 30-fold (data not shown).

We next analyzed nuclear and cytoplasmic poly(A)$^+$ p17$^{gag}$ mRNA levels in HLtat cells transfected with pCH16RRE in the absence or presence of Rev. Serial dilutions of extracted mRNAs were subjected to RT-PCR. The results revealed that high levels of p17$^{gag}$ mRNA were present in the cytoplasm in the absence of Rev (Fig. 8C). A two- to fourfold increase in both nuclear and cytoplasmic p17$^{gag}$ mRNA levels was observed in the presence of Rev (Fig. 8C). The amount of CAT protein produced from pCMVCAT included as an internal control for transfection efficiency varied less than twofold between transfections. The levels of spliced actin mRNA were similar in the absence or presence of Rev, demonstrating that similar amounts of poly(A)$^+$ mRNA were used for analysis (Fig. 8C). To control for the fractionation technique used to separate nuclei from cytosol, RT-PCR was performed with oligonucleotides specifically detecting unspliced actin mRNA. These results showed that unspliced actin RNA was found

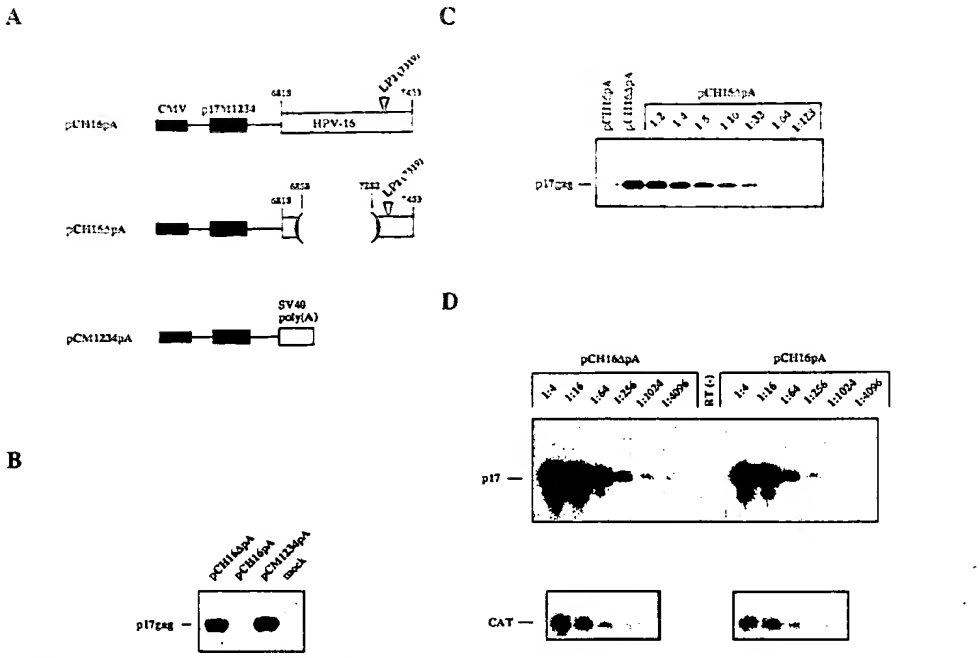


FIG. 7. Inhibitory sequences in the HPV-16 late 3' UTR. (A) Schematic structures of $p17^{gag}$ expression plasmids. Solid boxes indicate the human CMV immediate early promoter. Darkly shaded boxes indicate the mutated $p17^{gag}$ gene named p17M1234 (65). Open boxes indicate the HPV-16 late 3' UTR-containing sequences. The lightly shaded box indicates the SV40 early poly(A) signal. Brackets mark the limits of a deletion between nt 6858 and 7282. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 3' UTR and is shown as an open triangle (43, 60). The names of the plasmids are indicated on the left. (B) Western blot analysis of $p17^{gag}$ production from HeLa cells transfected with pCH16ΔpA, pCH16pA, or pCM1234. Proteins were resolved on SDS–15% polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes. The $p17^{gag}$ protein was detected with an HIV-1-positive patient serum, followed by incubation with horseradish peroxidase-conjugated anti-human immunoglobulin G antiserum. $p17^{gag}$ was visualized with enhanced chemiluminescence detection reagents. The position of the $p17^{gag}$ protein is indicated on the left. (C) Western blot analysis of $p17^{gag}$ production from cells transfected with pCH16ΔpA and pCH16pA, respectively. Cell lysate from pCHPV1ΔpA-transfected cells was serially twofold diluted, and $p17^{gag}$ protein was determined by Western immunoblotting as described in the legend to Fig. 7B. The position of $p17^{gag}$ protein is indicated on the left. (D) RT-PCR analysis of dilutions of cytoplasmic poly(A)$^+$ mRNA. Serial fourfold dilutions of cytoplasmic poly(A)$^+$ mRNAs from cells transfected with pCH16ΔpA or pCH16pA were reverse transcribed, followed by PCR. The upper panel shows PCR amplification of the cDNA samples with oligonucleotides specific for the $p17^{gag}$ sequence present in pCH16ΔpA and pCH16pA. The lower panel shows PCR amplification of the same cDNA samples with oligonucleotides specific for the CAT sequence present in the CAT expression plasmid used here to control for transfection efficiency. Oligonucleotides were end labeled with [$\gamma$-$^{32}$P]ATP prior to PCR. The PCR products were analyzed on 6% polyacrylamide gels, followed by autoradiography. The positions of the amplified DNA fragments are shown on the left. RT (−) indicates that RT-PCR was performed in the absence of RT in the cDNA synthesis reaction mixture.

primarily in the nuclear fraction (Fig. 8C). In conclusion, Rev had only a moderate effect on $p17^{gag}$ mRNA levels, while $p17^{gag}$ protein levels were greatly increased in the presence of Rev. Therefore, Rev and RRE acted primarily by increasing translation of the mRNAs containing inhibitory HPV-16 sequences.

**SRV-1 CTE counteracts the effect of inhibitory sequences in the HPV-16 late 3' UTR.** As shown above, the SRV-1 CTE element could efficiently overcome inhibition exerted by the inhibitory sequences in the HPV-16 L1 coding region. We also investigated whether the SRV-1 CTE could overcome inhibition exerted by the inhibitory sequences in the HPV-16 late 3' UTR. The SRV-1 CTE was inserted into pCH16pA in sense and antisense orientations, resulting in pCH16CS and pCH16CA, respectively (Fig. 9A). Figure 9B shows that pCH16CA produced low levels of $p17^{gag}$, while pCH16CS produced high levels of $p17^{gag}$, similar to those produced from pCM1234pA. These results demonstrated that the SRV-1 CTE element was able to counteract the negative effect of the HPV-16 late 3' UTR sequences.

We also analyzed cytoplasmic poly(A)$^+$ mRNA levels in HLtat cells transfected with pCH16CS or pCH16CA. RT-PCR was performed on serially fourfold-diluted RNA samples with oligonucleotides specific for $p17^{gag}$ mRNA. The results revealed that approximately four times more $p17^{gag}$ mRNA was present in cells transfected with pCH16CS than in cells transfected with pCH16CA (Fig. 9C). Actin mRNA levels in these two samples were similar, demonstrating that similar amounts of poly(A)$^+$ mRNA were used in the RT-PCR. Quantitation of serially diluted $p17^{gag}$ protein by Western blot analysis revealed that pCH16CS produced approximately 64-fold higher levels of $p17^{gag}$ than did pCH16CA (data not shown). Therefore, the presence of the CTE element on the mRNA resulted in increased cytoplasmic poly(A)$^+$ mRNA levels and increased translation of mRNAs containing inhibitory HPV-16 sequences.

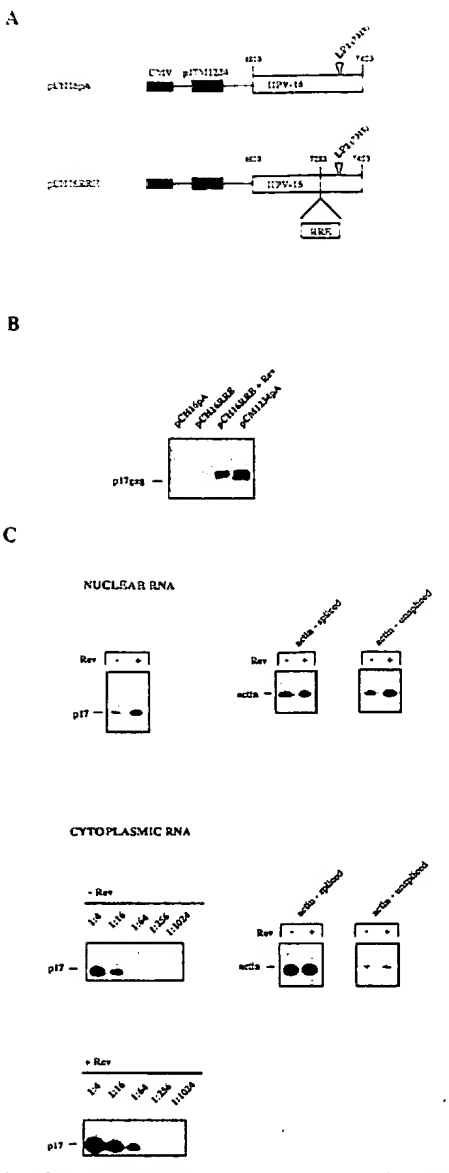


FIG. 8. Comparison of p17$^{mut}$ protein levels and nuclear and cytoplasmic mRNA levels from cells transfected with pCH16RRE in the absence or presence of Rev protein. (A) Schematic structures of p17$^{mut}$ expression plasmids. Solid boxes indicate the human CMV immediate early promoter (CMV), shaded boxes indicate the mutated p17$^{mut}$ gene named p17M1234 (65), and open boxes represent the HPV-16 late 3′ UTR-containing sequences or the HIV-1 RRE as indicated. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 3′ UTR and is shown as an open triangle (43, 60). The names of the plasmids are displayed on the left. (B) Western blot analysis of extracts of cells transfected with pCH16pA, pCM1234, or pCH16RRE in the absence or presence (+) of Rev protein. p17$^{mut}$ production was analyzed by Western immunoblotting as described in the legend to Fig. 7B. The position of p17$^{mut}$ protein is indicated on the left. (C) RT-PCR analysis of nuclear and cytoplasmic poly(A)$^+$ mRNA. The upper left panel shows PCR amplification of the cDNA synthesized from poly(A)$^+$ mRNAs isolated from nuclear fractions of cells transfected with pCH16RRE in the absence or presence of Rev protein. Oligonucleotides were end labeled with [$\gamma$-$^{32}$P]ATP prior to PCR. The upper right panel shows PCR amplification of cDNA from spliced or unspliced actin mRNAs in the nuclear fraction of cells transfected with pCH16RRE in the absence or presence of Rev protein. The positions of the amplified DNA fragments are indicated on the left. The lower left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)$^+$ mRNAs isolated from cells transfected with pCH16RRE in the absence or presence of Rev protein. The lower right panel shows PCR amplification of cDNA from spliced or unspliced actin mRNAs in the cytoplasmic fraction of cells transfected with pCH16RRE in the absence or presence of Rev protein.

Transcription in the cytoplasm relieves inhibition exerted by sequences in the HPV-16 late 3′ UTR. The results described above showed that inhibition exerted by the negative elements in the HPV-16 L1 coding sequence could be relieved if transcription of these sequences occurred in the cytoplasm. We examined if this was also true for the inhibitory sequences in the HPV-16 late 3′ UTR. The CMV promoter present in pCH16pA and pCH16ΔpA was replaced with the bacteriophage T7 promoter, resulting in plasmids pT7-16pA and pT7-16ΔpA (Fig. 10A), respectively. HeLa cells were infected with the recombinant vaccinia virus vTF7-3 (27, 28), producing T7 RNA polymerase, followed by transfection with pT7-16pA or pT7-16ΔpA. Western blot analysis revealed that similar high levels of p17$^{mut}$ protein were produced from both plasmids (Fig. 10B), demonstrating that transcription in the cytoplasm relieves inhibition exerted by the inhibitory element in the HPV-16 late 3′ UTR. These results indicate that nuclear factors are required for inhibition.

## DISCUSSION

In this study, we show that the HPV-16 major capsid protein L1 coding region contains intragenic inhibitory sequences that act in an orientation-dependent manner to posttranscriptionally inhibit L1 expression. We also confirmed previous findings (44) that an independently acting inhibitory element is present in the HPV-16 late 3′ UTR. The presence of these inhibitory elements on HPV late mRNAs offers an explanation for previous observations demonstrating that expression of HPV-16 capsid proteins is restricted to terminally differentiated keratinocytes. Interestingly, production of HPV-16 L1 could be activated with HIV-1 Rev and RRE or by providing the SRV-1 CTE element. Alternatively, inhibition could be bypassed by transcription of the HPV-16 L1 sequence in the cytoplasm with the vaccinia virus-T7 RNA polymerase-based expression system (27, 28), suggesting that nuclear processes participate in the inhibition.

Similarly to the HPV-16 L1 mRNAs, HIV-1 mRNAs encoding Gag, Pol, and Env proteins contain inhibitory sequences in coding regions (10, 14, 51, 57, 62, 65, 67). These sequences prevent expression of viral structural proteins in the absence of Rev. Comparison of inhibitory sequences in coding regions of HIV-1 *gag* with those in HPV-16 L1 mRNAs revealed that they have a high AU content. One inhibitory element on the HIV-1 *gag* mRNA was characterized in detail in previous work as an AU-rich sequence with an AU content of 61.5% (65, 67). The AU content of the HPV-16 L1 region associated with inhibitory sequences (nt 5813 to 6150) is 59.6%. Similarly, the previously identified negative elements in the HPV-1 and HPV-16 late 3′ UTRs were described as AU-rich sequences (44, 72). AU richness may be a general property of viral RNA se-

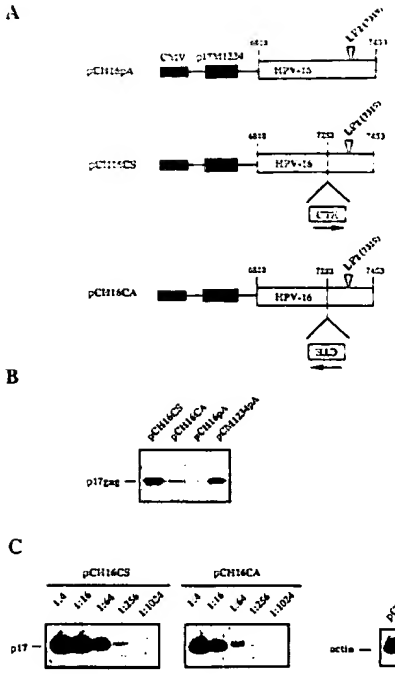


FIG. 9. Inhibition exerted by the HPV-16 late 3' UTR is relieved by the presence of the SRV-1 CTE in the sense orientation. (A) Schematic structures of $p17^{gag}$ expression plasmids. The solid boxes indicate the human CMV immediate early promoter (CMV), shaded boxes indicate the mutated $p17^{gag}$ gene named p17M1234 (65), and open boxes represent the HPV-16 late 3' UTR-containing sequences or the SRV-1 CTE-containing sequence as indicated. Arrows indicate the orientation of the CTE in the plasmids. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 3' UTR and is shown as an open triangle (43, 60). The names of the plasmids are displayed on the left. (B) Western blot analysis of extracts from cells transfected with pCH16CS, pCH16CA, pCH16pA, or pCM1234pA. The production of $p17^{gag}$ was analyzed by Western immunoblotting as described in the legend to Fig. 7B. The position of $p17^{gag}$ protein is indicated on the left. (C) RT-PCR analysis of dilutions of cytoplasmic poly(A)$^+$ mRNA. The left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)$^+$ mRNAs isolated from cells transfected with pCH16CS or pCH16CA. Oligonucleotides were end labeled with [$\gamma$-$^{32}$P]ATP prior to use. The right panel shows PCR amplification of cDNA synthesized from spliced actin mRNAs in the cytoplasmic fraction of cells transfected with pCH16CS or pCH16CA. The positions of the amplified DNA fragments are indicated on the left.

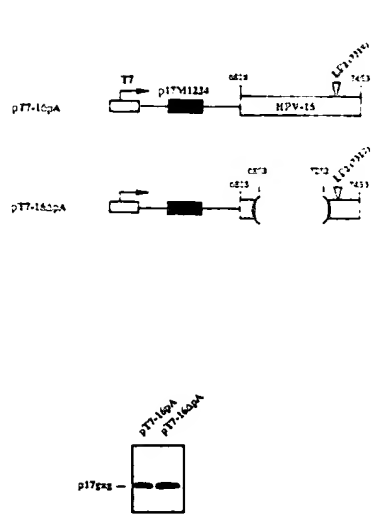


FIG. 10. Transcription in the cytoplasm could bypass inhibition exerted by the inhibitory element in the HPV-16 late 3' UTR. (A) Structures of the $p17^{gag}$ expression plasmids. Small open boxes indicate the bacteriophage T7 promoter, and large open boxes indicate HPV-16 sequences. Shaded boxes indicate the mutated $p17^{gag}$ gene named p17M1234 (65). Bent arrows indicate the direction of transcription driven by the T7 promoter. The brackets mark the limits of a deletion between nt 6858 and 7282. The numbers indicate nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 late 3' UTR and is shown as an open triangle (43, 60). The names of the plasmids are indicated on the left. (B) Western blot analysis of $p17^{gag}$ production. HeLa cells were infected with recombinant vaccinia virus vTF7-3 (27, 28), followed by transfection with pT7-16pA or pT7-16ΔpA. Production of $p17^{gag}$ was analyzed by Western immunoblotting as described in the legend to Fig. 7B. The position of $p17^{gag}$ protein is indicated on the left.

quences with inhibitory function. In addition, several cellular mRNAs that are under posttranscriptional regulation contain AU-rich inhibitory sequences in the 3' UTR. Such AU-rich sequences usually contain multiple copies of the AUUUA pentanucleotide (13, 63). This sequence motif is not present in the negative element in the HPV-16 late 3' UTR (44). However, the inhibitory region in the HPV-16 L1 coding sequence (nt 5813 to 6150) contains seven AUUUA pentanucleotides. Additional experiments are required to establish the role of AUUUA motifs in HPV-16 L1 posttranscriptional inhibition. The various sequence motifs proposed by Kennedy et al. to play a role in the inhibitory function of the negative element in the HPV-16 late 3' UTR (44) were not present in the L1 inhibitory coding region. In bovine papillomavirus type 1, splice site-like sequences have been proposed to play a regulatory role in late gene expression (29). Sequences with substantial homology to the 5' splice site consensus sequence AAGGUAAGU could not be found in the HPV-16 L1 inhibitory region (nt 5813 to 6150), while the HPV-16 late 3' UTR contains 5' splice site-like sequences. It has been suggested that these sequences inhibit HPV-16 late gene expression (29). It has been reported that unutilized splice sites on HIV-1 mRNAs inhibit late gene expression in the absence of Rev (11, 49). This is not easy to reconcile with the fact that *gag* and *pol* mRNAs produced from simple retroviruses lacking *rev* genes also contain unutilized splice sites but are efficiently exported to the cytoplasm. There is also growing evidence that sequences distinct from splice sites inhibit expression of HIV-1 late genes in the absence of Rev (10, 57, 65). If such sequences are altered by mutagenesis, production of the HIV-1 late gene products occurs in the absence of Rev (65).

Although the function of HIV-1 Rev has been extensively studied, the exact mechanism of Rev regulation is not fully elucidated. It is generally accepted that Rev facilitates transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm (17, 25, 26, 36, 59). In addition, Rev has been proposed to increase mRNA stability (25, 52, 67) and translatability (2, 18, 48). Our results on HPV expression reported here revealed that high levels of L1 mRNA produced from plasmid p16L1 were present in the cytoplasm in the absence or

presence of Rev, while L1 protein was only detectable in the presence of Rev. When Rev protein was provided in *trans*, cytoplasmic L1 mRNA levels produced from p16L1RRE increased less than fourfold (Fig. 2E), while high levels of L1 protein were produced (Fig. 2C). This demonstrated that HPV-16 L1 mRNAs were produced and exported from the nucleus in the absence of Rev. However, these mRNAs were not efficiently translated, indicating that the interaction of Rev and RRE improves utilization of RRE-containing mRNAs. Similar results were obtained when expression of CAT mRNA and protein levels in cells transfected with pNLCATW or pCATL1SS, which contains HPV-16 L1 inhibitory sequences, were compared. There is only a 4-fold difference between the levels of cytoplasmic CAT mRNA produced from these two plasmids, while the difference in CAT protein levels was greater than 140-fold. It is possible that in the absence of Rev protein, the HPV-16 L1 mRNAs are transported to a cellular compartment in which they are not accessible to the cellular translation machinery. Our results are consistent with those of previous studies showing that the Rev protein has a positive effect on translation efficiency by affecting mRNA loading on polysomes (2, 18, 48). To explain these results, we postulate that the route by which mRNAs are transported from the nucleus to the cytoplasm or the cellular factors associated with the mRNA may determine the fate of mRNAs in the cytoplasm. Rev may efficiently interact with RRE-containing mRNAs by helping them enter a productive nuclear export pathway used by cellular mRNAs, thereby directing them to the cellular translation machinery. Alternatively, Rev may compete with a nuclear factor that interacts with the inhibitory sequences and acts by inhibiting RNA processing or translation. Therefore, Rev may indirectly affect translation of RRE-containing mRNAs. It is reasonable to assume that events which decrease utilization of the HPV-16 L1 mRNA occur in the nucleus before the mRNA enters the cytoplasm, since transcription of HPV-16 L1 coding sequences in the cytoplasm with a vaccinia virus-T7 RNA polymerase-based expression system resulted in loss of inhibition.

In addition to the HIV-1 Rev-RRE system, the SRV-1 CTE could activate expression of HPV-16 L1. The CTE element does not require the presence of viral *trans*-acting factors for function, indicating that a Rev-like cellular factor interacts with this sequence to overcome the inhibitory effect of the negative elements on the HPV-16 L1 mRNAs. Since a positive element similar in function to the SRV-1 CTE has not been identified on the late HPV-16 mRNAs, it is conceivable that cellular factors which bind to the HPV-16 inhibitory element are present in basal cells of the epithelium, while expression or activity of such cellular factors is inhibited in terminally differentiated keratinocytes. Alternatively, positive cellular or viral regulatory factors able to compete with negative factors bound to inhibitory RNA sequences are activated in fully differentiated keratinocytes. Since a Rev-like protein has not been identified in any known HPV, we favor a model which predicts that cellular negative factors bound to inhibitory HPV sequences are inactivated as epithelial cells differentiate. This would allow expression of HPV late gene products.

Although inhibitory elements present in the HPV-16 L1 coding sequence and HPV-16 late 3′ UTR are structurally different and functionally independent, they act in parallel to reduce polyadenylated mRNA levels and to inhibit translation of mRNA. The fact that multiple inhibitory elements are present on the HPV-16 late mRNAs indicates that the presence of these elements may be important to ensure that viral L1 protein is produced only when infected cells differentiate and reach the superficial layer of the epithelium. Production of virus in terminally differentiated keratinocytes may be important for efficient spreading of virus or for the avoidance of an immune reaction against HPV-producing cells. Similarly, HIV-1 mRNAs contain multiple inhibitory elements that prevent expression in the absence of Rev. Also c-*myc* (6), c-*fos* (42, 69, 74), and urokinase-type plasminogen activator (56) mRNAs are found to contain multiple inhibitory elements, suggesting that this is a general feature of posttranscriptionally regulated mRNAs.

Although HPV-16 and HIV-1 are not related, the negative effect on gene expression exerted by the HPV-16 L1 inhibitory element could be overcome by HIV-1 Rev and RRE. This is a surprising finding which indicates that HIV and HPV may use a similar strategy to regulate expression of late genes. However, HIV-1 late mRNAs are located primarily in the nucleus in the absence of Rev, while the HPV-16 L1 mRNAs are located in the cytoplasm but are not translated. This may imply a different kind of regulation. The results presented here support the idea that Rev is a positive regulator able to correct different defects on viral or cellular mRNAs at a posttranscriptional level. In agreement with this, it was recently shown that the effect of a cellular mRNA instability element in the 3′ UTR of the transferrin receptor mRNA could be overcome by the Rev-RRE regulatory system of HIV-1 or by the Rex-RexRE system of human T-cell leukemia virus type 1 (75).

The finding that CTE or Rev and RRE can activate HPV-16 L1 expression in human epithelial cells may have practical applications. HPVs cannot be propagated in vitro, presumably as a result of a block in late gene expression in cells other than terminally differentiated keratinocytes. A genomic HPV-16 clone containing CTE inserted in the late region may produce virus upon introduction into human cells. Furthermore, HPV-16 L1 protein has been shown to self-assemble into viruslike particles in insect cells with a baculovirus-based expression system (46). Viruslike particles have proved to be a more specific antigen than denatured particles in serological screenings of HPV-infected individuals (45). It will be of interest to investigate if HPV-16 L1 produced with the posttranscriptional regulatory systems of HIV-1 and SRV-1 assembles into viruslike particles in human cells and if such viruslike particles induce strong immune responses against HPV-16.

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# *Cis*-Acting Negative RNA Elements on Papillomavirus Late mRNAs

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**Papillomaviruses comprise a large number of related small DNA tumor viruses with tropism for squamous epithelial cells. The papillomavirus replication cycle is strictly linked to the differentiation stages of the infected epithelial cells, and the expression of L1 and L2 capsid proteins from the viral late genes is primarily detected in the superficial layers of terminally differentiated cells. Expression of the L1 and L2 genes is blocked in nonterminally differentiated cells and the production of progeny virus is delayed until the infected cell reaches the upper strata of the squamous epithelium. This property presumably aids the papillomavirus to evade the immune surveillance of the host and allows establishment of persistent infections. Late gene expression levels are determined in part by regulatory RNA sequences on the papillomavirus mRNAs. This review focuses primarily on negative *cis*-acting elements on late papillomavirus mRNAs and their candidate *trans*-acting factors. Identification and characterization of these components will contribute to our understanding of the regulated expression of papillomaviruses in mammalian cells.** © 1998 Academic Press

***Key Words:*** **CTE; L1; L2; papillomavirus; posttranscriptional gene regulation; Rev.**

## PAPILLOMAVIRUS LIFE CYCLE

The papillomavirus family is remarkably heterogeneous and includes a large number of related epitheliotropic, small DNA viruses (1, 2). They are associated with a variety of neoplasias and include important human pathogens that are found in anogenital cancers and skin cancers (2, 3). All papillomaviruses contain a circular double-stranded DNA genome of approximately 8 kb that is contained as a multicopy episome in the infected cell (1, 4, 5). The genome can be divided into three regions encoding early genes, late genes, and a noncoding region (NCR) (Fig. 1). The early genes express proteins involved in transcriptional regulation, virus DNA replication, and cell transformation, whereas the late genes code for the major and minor capsid proteins L1 and L2, respectively (1, 2) (Fig. 1). Together with a circular copy of the virus DNA genome, the L1 and L2 proteins assemble into icosahedral infectious virions with a diameter of approximately 55 nm (6).

The papillomavirus life cycle is intricately linked to the cell differentiation program and completion of the virus replication cycle is dependent on terminal differentiation of the infected epithelial cell (1, 5, 7, 8). Papillomaviruses infect cutaneous or mucosal epithelia and it is believed that infection occurs as a result of microtraumas that expose cells in the basal layers of the squamous epithelium to virus entry (1). The virus early genes are expressed in all layers of the epithelium (Fig. 2) and produce a large number of multiply spliced mRNAs which direct synthesis of the early proteins (9). These mRNAs are polyadenylated at the early poly(A) signal located immediately upstream of the late region on the virus genome (Fig. 1). Production of the late proteins L1 and L2 is blocked in cells in the lower layers of the epithelium and is restricted to the terminally differentiated cells in the upper layers of the epithelium (1, 5, 7, 8) (Fig. 2). Apparently, the blockage of L1 and L2 expression is released or overcome as the infected cell enters the granular or most differentiated epithelial cell layers (Fig. 2). One consequence of this is that *in vitro* propagation of papillomavirus has been successful only when the infected or transfected epithelial cells are allowed to differentiate in organotypic cell cultures or have been transplanted into nude mice (7, 10, 11).

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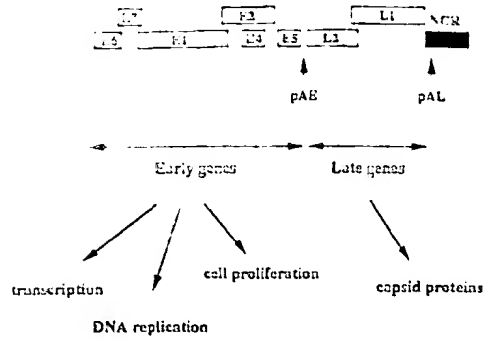


FIG. 1. Generic papillomavirus genome. Open boxes indicate open reading frames encoding early (E) and late (L) genes and the black box indicates the noncoding region (NCR). pAE and pAL, early and late poly(A) signals, respectively

Papillomavirus late gene expression is tightly controlled and regulated at both transcriptional and posttranscriptional levels (1). Differentiation-dependent promoters that are induced to produce papillomavirus late mRNAs polyadenylated at the late poly(A) signals downstream of the L1 open reading frame (Fig. 1) have been identified (12–14). *Cis*-acting DNA elements that are required for differentiation-dependent transcription may be identified by analysis of the transcriptional activity of the NCR region in raft cultures with differentiating cells (15). In addition, late gene expression can be regulated at the posttranscriptional level. The identification of negative, regulatory RNA sequences on papillomavirus late mRNAs suggest that these sequences are important determinants of papillomavirus L1 and L2 expression levels (16–19). Our current knowledge of the papillomavirus inhibitory RNA sequences and their putative *trans*-acting factors is summarized below

## REGULATORY RNA SEQUENCES ON CELLULAR mRNAs

Cellular and viral mRNAs contain sequences that modulate the levels of protein produced from the expressed gene. Regulatory RNA sequences are located in the 5′ untranslated region (UTR), in the protein coding sequence, or in the 3′ UTR of the mRNA and possibly act by targeting the mRNAs to premature degradation or by prevention of translation (20). The mRNA half lives and translation efficiencies are not static but fluctuate in response to environmental factors. The inhibitory RNA sequences are believed to interact with cellular *trans*-acting factors that determine the fate of the mRNAs. It is possible that the RNA binding activity of such factors may change in response to certain cell stimuli, thereby increasing or decreasing the levels of the target gene product. Well characterized *cis*-acting RNA elements include the iron response element in the 5′ UTR on the ferritin mRNA which interacts with the iron response element binding protein that imposes a translational block on the mRNA under conditions of low concentration of intra-

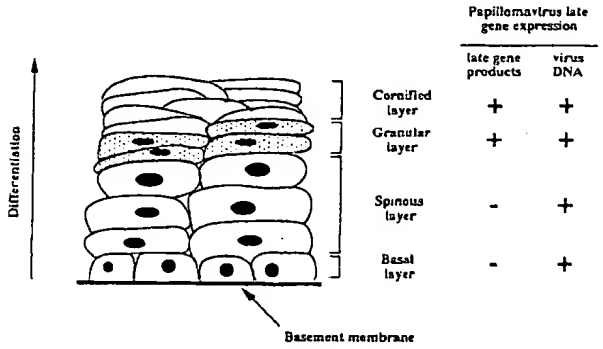


FIG. 2. Schematic diagram of stratified epithelium. Cells in the basal layer are dividing, resulting in one cell that retains the ability to divide and one daughter cell that migrates upward, entering the terminal differentiation program. Papillomavirus DNA can be detected in all layers whereas the late gene products L1 and L2 are detected primarily in stratum granulosum and corneum.

cellular iron (21). The mRNA 3′ UTR may also contain regulatory RNA sequences. Among the best characterized of these sequences are the histone mRNA 3′ UTR element (22), the transferrine receptor mRNA 3′ UTR element (21), and the AU-rich RNA elements (AREs) (20, 23). The latter elements have been found on a large number of cellular mRNAs that are under posttranscriptional control (20). Although the exact definition of an ARE is not clear, it appears that the majority of these elements contain AUUUA pentamers in an AU-rich environment and target mRNAs for rapid degradation, presumably a result of their interactions with cellular factors (20). For example, the c-fos ARE can be divided into two domains, each shorter than 50 nt and containing either AUUUA pentamers or U-rich stretches (24). Regulatory elements have also been located to the mRNA coding regions on, for example, c-myc and c-fos mRNAs, but these sequences are in general less well characterized than the 3′ UTR elements (20). In conclusion, many cellular mRNAs contain *cis*-acting mRNA elements that interact with cellular factors that determine the fate of the synthesized mRNA. It is likely that viruses take advantage of the posttranscriptional regulatory machinery present in the cell to modulate expression of their own genes. Therefore, identification and characterization of virus *cis*-acting regulatory RNA sequences and their *trans*-acting cellular factors may contribute to our understanding of posttranscriptional regulation of virus and cellular gene expression.

## INHIBITORY RNA SEQUENCES ON HUMAN PAPILLOMAVIRUS TYPE 1 LATE mRNAs

Human papillomaviruses (HPVs) are strictly epitheliotropic and different HPV types preferentially infect mucosal or cutaneous epithelium at certain anatomical sites (3). HPV-1 is tropic for cutaneous epithelium and is the etiologic agent of deep plantar warts. Such warts are benign tumors and usually regress within 2 years, presumably as a result of cell-mediated immune responses against virus-infected cells. Immunostainings of HPV-1-infected epithelium have established that the HPV-1 L1 and L2 proteins are detected primarily in the upper layers of terminally differentiated epithelial cells (25), a differentiation-dependent late gene expression pattern typically observed in HPV-infected squamous epithelial cells. HPV-1 virions are found in abundance throughout infected cells (26) to such an extent that experiments can be performed on primary cell cultures (27) or skin from human hand or foot (28).

One explanation for the absence of HPV-1 late gene expression in the lower layers of nonterminally differentiated cells is the presence of inhibitory sequences on late HPV-1 mRNAs. To identify negative elements, certain sequences spanning various regions of the late HPV-1 mRNAs were inserted after a reporter gene in eucaryotic expression plasmids and the effect on the reporter gene expression was determined in transient transfections (18). The results revealed the existence of inhibitory sequences in the HPV-1 late 3′ UTR and indicated that they decreased expression levels of the reporter gene in an orientation-dependent manner (18, 29, 30) (Fig. 3). In contrast, the 3′ UTR of the early mRNAs did not inhibit gene expression in the same assay (29) (Fig. 3), demonstrating that inhibitory activity is confined to the late mRNAs. The inhibitory sequence acts *in cis* to reduce the mRNA half life and prevents efficient utilization of the mRNA in the cell (18, 30).

The HPV-1 inhibitory element has been mapped to a 57-nt sequence located immediately downstream of the L1 stop codon (30) (Fig. 4). This sequence contains 93% A + U and can be divided into two parts; a 5′ region that contains two AUUUA motifs and a 3′ region that contains three UUUUU repeats (Fig. 5A and Table 1). Deletions or point mutations in AUUUA or UUUUU motifs reduced the inhibitory activity, whereas point mutations in both motifs abolished the inhibitory activity (30). AUUUA and UUUUU motifs are hallmarks of many AU-rich mRNA instability elements located in the 3′ UTRs of a variety of labile cellular mRNAs, typically those encoding proto-oncogenes and cytokines (20, 24). The similarity between the c-fos and the HPV-1 AU-rich elements is particularly strong as both elements contain two AUUUA and three UUUUU motifs in an AU-rich context (Fig. 5A) (18) that act by reducing mRNA stability (30).

Multiple nuclear and cytoplasmic proteins in HeLa cells interact with the HPV-1 AU-rich inhibitory sequence (29) (Fig. 5B). All proteins are characterized by having high affinity for poly(U) while they show weak or no binding to other homoribopolymers (29, 30). Of particular interest are three proteins with molecular masses of 38, 44, and 46 kDa since they do not bind to the 3′ UTR of early HPV-1 mRNAs and they fail to interact with HPV-1 RNAs with point mutations that reduce the inhibitory activity of the AU-rich sequence (29, 30). The 44- and 46-kDa proteins were identified as heterogenous nuclear ribonucleoproteins (hnRNP) C1

and C2 (Fig. 5B and Table 1) (30). hnRNP C1 and C2 have been found to bind to U-rich sequences of the poly(A) signal and to polypyrymidine-rich intron sequences on cellular mRNAs, but their role in polyadenylation or splicing remains unclear (31). The 38-kDa HPV-1 RNA binding protein binds to UUUUU motifs and appears to share many characteristics with the CRBP protein that interacts with the UUUUU sequences in the c-fos RNA instability element (32). A compelling fact is that all proteins that interact with the HPV-1 AU-rich sequence also bind to the c-fos AU-rich element (30) (Fig. 5B). Interestingly, the expression patterns in squamous epithelium of HPV-1 capsid proteins and c-fos protein overlap and show strong staining in terminally differentiated cells in the superficial cell layers of the epithelium (33), strongly suggesting that expression of the late HPV-1 genes and cellular c-fos are under control of the same regulatory machinery (30).

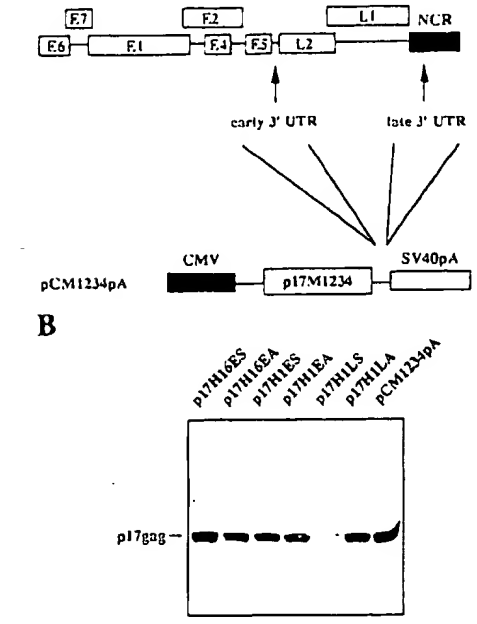


FIG. 3. Inhibitory sequences are present in the 3' UTR of late but not early HPV-1 and HPV-16 mRNAs. (A) The 3' UTR sequences of HPV-1 and HPV-16 early or late mRNAs were inserted in sense or antisense orientation downstream of the p17M1234 reporter gene (29). (B) Plasmids were transfected into HeLa cells and reporter gene expression was monitored by Western immunoblotting. The results show that HPV-1 and HPV-16 early 3' UTR sequences lack inhibitory activity, whereas the HPV-1 late 3' UTR contains sequences that inhibit gene expression in an orientation-dependent manner (29). Reproduced from reference 29 with permission of the publisher.

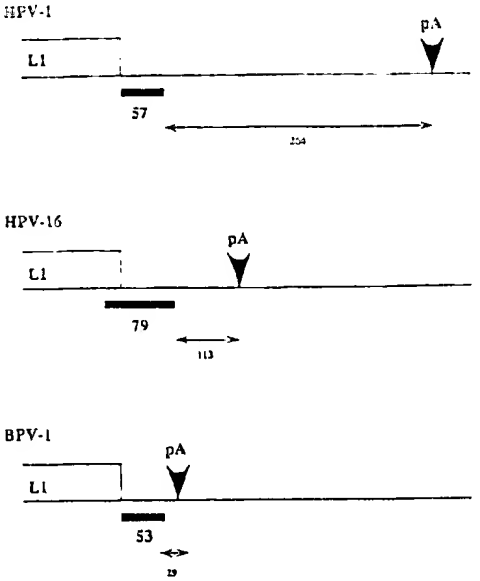


FIG. 4. Schematic diagram of the late 3' UTR region of HPV-1, HPV-16, and BPV-1 (16–18, 37). Black bars indicate the location of negative RNA elements and numbers indicate their sizes in nucleotides. The distance between the negative element and the poly(A) signal is shown below the double headed arrow. HPV-1 pA refers to pA1 (18) and HPV-16 pA refers to L?2 (37).

The interaction of the same cellular proteins with HPV-1 and c-fos RNA instability determinants may have significant implications on the pathogenesis of HPV infection. Each infected cell contains a large number of HPV genomic copies that have the potential to direct synthesis of mRNAs, including late mRNAs containing the inhibitory RNA sequences. Inhibitory HPV-1 mRNA sequences may compete with the c-fos mRNA instability elements for cellular RNA binding proteins, which in turn may unblock the restriction imposed on c-fos expression by these factors. Deregulated c-fos production is a property of many tumor cells. In addition, it has been shown that deletion of the 3' UTR sequence encoding the mRNA instability deter-

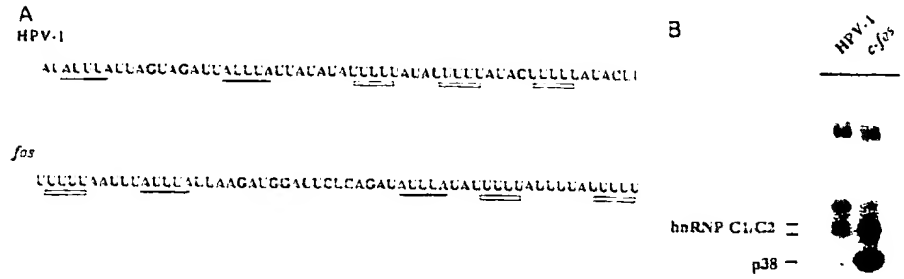


FIG. 5. (A) Primary sequence of the HPV-1 and the c-*fos* AU-rich elements. AUUUA and UUUUU motifs are underlined. (B) UV-cross linking of HeLa cell nuclear proteins to *in vitro* synthesized, radiolabelled HPV-1 or c-*fos* AU-rich RNA elements (29, 30).

minant of the c-fos mRNA increased the oncogenicity of the c-fos gene (34, 35), demonstrating that deregulated posttranscriptional gene regulation may contribute to uncontrolled cell growth and tumorogenesis.

## INHIBITORY RNA SEQUENCES ON HUMAN PAPILLOMAVIRUS TYPE 16 LATE mRNAs

HPV-16 is a sexually transmitted genital HPV type with tropism for mucosal epithelial cells which may establish persistent infections that in rare cases progress to cancer (3). The time span between infection and invasive cancer can be several decades (3). Apparently, HPV-16 has evolved to evade the immune responses of the host. Low production of virions in infected cells and restriction of virus production to the upper layers of the epithelium are probably important properties that enables the virus to persist in the infected host. HPV-16 late mRNAs contain inhibitory sequences that may contribute to the regulated expression of the L1 and L2 late genes (17, 19, 36).

### Negative elements in the HPV-16 late 3' UTR

Early work identified an inhibitory region located at the HPV-16 L1 stop codon and extending into the late 3' UTR (Fig. 4) (17, 37). Plasmids designed to express the bacterial CAT gene showed reduced CAT levels when 3' UTR sequences and poly(A) signals were derived from the HPV-16 late mRNA region compared with SV40. The inhibitory HPV-16 region mapped to a 79-nt sequence (nt position 7128 to 7206 in the HPV-16 genome) (17, 37, 38). This region could be further divided into a 5' portion containing four 5' splice-site-like sequences and a 3' portion with a high GU content (Table 1) (36, 38). In contrast to HPV-1, the inhibitory sequence in the HPV-16 late 3' UTR does not encode AUUUA or UUUUU motifs. However, *in vitro* synthesized HPV-16 RNA encoding the inhibitory sequence was rapidly degraded *in vitro* after incubation with polysomes (17), suggesting that this sequence may target HPV-16 late mRNAs for rapid degradation. A 51-nt sequence spanning the 5' portion of the HPV-16 negative element has been studied using site-specific mutagenesis. In this sequence, the major inhibitory activity coincided with a 5' splice-site-like sequence (Table 1), and point mutations in the GU dinucleotide of this 5' splice-site-like sequence abolished inhibitory activity (36). In the context of its natural poly(A) signal, mutations in the same 5' splice-site-like sequence reduced inhibitory activity, but the mutant HPV-16 sequences retained most of their ability to inhibit gene expression (38), demonstrating that the downstream

TABLE 1
Negative *Cis*-Elements on Papillomavirus Late mRNAs and Their Putative *Trans*-Acting Factors

| Papillomavirus | *Cis*-elements | *Trans*-factors |
|---|---|---|
| HPV-1 | UUUUU<br>AUUUA | hnRNPC1/C2, p38 |
| HPV-16 | G + U rich stretch | U2AF65 |
| | GCUGUAAG | U1 snRNA |
| BPV-1 | AAGGUAAGU | U1 snRNA |

G+U rich region and the 5' splice-site-like sequence act independently and are both required for efficient inhibitory activity.

A 65-kDa protein believed to be U2AF65 interacts with the GU-rich sequence in the HPV-16 late 3' UTR (39) (Table 1). Through mutations that were expected to reduce binding of U2AF65, the binding activity of the 65-kDa was shown to be weaker. The results suggested that binding of this protein to the HPV-16 RNA element was required for inhibition. Proteins binding to the 5' portion encoding the 5' splice-site-like sequences were not detected using UV cross linking (38). However, it has been suggested that this sequence interacts with U1 small nuclear RNA and that this interaction results in reduced gene expression (36).

### Negative elements in the HPV-16 L1 and L2 coding regions

The HPV-16 L1 and L2 coding regions contain inhibitory RNA sequences that act independently of the negative elements located in the late 3' UTR (Fig. 6) (19, 60). In contrast to the papillomavirus late 3' UTR negative elements that appear to be encoded by sequences shorter than 79 nt, the L1 and L2 negative elements encompass larger regions (Fig. 6). Inhibitory activity of the sequences in L1 and L2 is probably dependent on the integrity of a large RNA region (19). For example, a negative element in the 5' end of HPV-16 L1 spans at least 600 nt (19), whereas one inhibitory region in HPV-16 L2 encompasses 800 nt (60). Further work will identify the *cis*-acting negative elements in HPV-16 L1 and L2 coding regions and their *trans*-acting factors.

## INHIBITORY RNA SEQUENCES ON BOVINE PAPILLOMAVIRUS TYPE 1 LATE mRNAs

Bovine papillomavirus type 1 (BPV-1) induces fibropapillomas in cattle and transforms primary bovine fibroblasts without need for additional oncogenes (39). Infection with BPV-1, purified from bovine fibropapillomas, can induce morphological transformation of established cell lines *in vitro*, e.g., NIH-3T3 and C127 murine cells (39). However, progeny virus is not released. Similarly to human papillomaviruses, production of BPV-1 virions is inherintly linked to the differentiation stage of the infected keratinocyte.

A 53-nt sequence located immediately upstream of the poly(A) signal in the BPV-1 late 3' UTR (Fig. 4) acts in an orientation-dependent manner to reduce the levels of cytoplasmic, polyadenylated mRNAs (16). Deletion of this sequence from a BPV-1 L1 cDNA resulted in increased levels of L1 mRNAs in the cytoplasm. Intriguingly, the cytoplasmic half life of the L1 mRNA was not altered following deletion of the inhibitory sequence (16), suggesting a mode of action other than targeting the BPV-1 late mRNAs for rapid RNA degradation in the cytoplasm and suggesting that the presence of this sequence reduced the polyadenylation efficiency of the late mRNAs (16). Further experiments revealed that the inhibitory activity of this region coincided with a 9-nt sequence, AAGGUAAGU, with perfect homology to the consensus 5' splice site (C/A)AGGU(A/G)AGU (36) (Table 1). This sequence alone showed stronger inhibition than the 53-nt sequence. The integrity of the 5' splice-site-like sequence was required since single point mutations in the invariable GU dinucleotide abolished inhibition (36). In general, mutations that are known to block splicing also inactivated the inhibitory sequence. Expression of

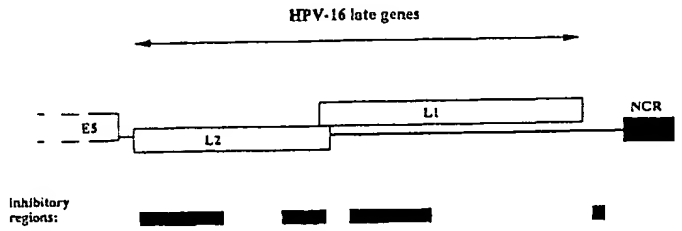


FIG. 6. The late region of the HPV-16 genome. Black bars indicate the location of inhibitory sequences in the L1 and L2 coding regions (19, 60).

a mutant U1 snRNA containing a compensatory mutation which allowed base pairing to the inactive inhibitory BPV-1 sequence carrying a mutation at position +5 in the 5' splice-site-like sequence partially restored inhibition, indicating that base pairing between the BPV-1 5' splice-site-like sequence and U1 snRNA was required for inhibitory activity of the BPV-1 sequence (Table 1) (36). Similarly to the G+U rich region in the inhibitory element in the HPV-16 late 3' UTR, the BPV-1 negative element may also interact with a splicing factor.

## HETEROLOGOUS POSTTRANSCRIPTIONAL REGULATION SYSTEMS CAN ACTIVATE PAPILLOMAVIRUS LATE GENE EXPRESSION

Similarly to papillomaviruses, expression of retroviruses is regulated at both transcriptional and posttranscriptional levels. The prototypic complex retrovirus human immunodeficiency virus type 1 (HIV-1) encodes two regulatory proteins named Tat and Rev (40–43). Tat activates transcription of the virus genome whereas the Rev protein binds to an RNA sequence named the Rev-responsive element (RRE) located on HIV-1 late mRNAs and promotes nuclear export and utilization of RRE-containing mRNAs (43). Rev and RRE are essential for completion of the virus replication cycle, but it was recently shown that virus production could be restored from a Rev and RRE deficient virus by insertion of a positive regulatory RNA sequence present in Mason-Pfizer monkey virus and simian retrovirus type 1, suggesting the existence of a cellular Rev-like protein (44). This sequence is known as the constitutive transport element (CTE) (45, 46). Interestingly, Rev and RRE or CTE can overcome the negative effect of the 3' UTR sequences of HPV-1 (18) and HPV-16 (19). Similarly, Rev and RRE counteract the negative effect of the BPV-1 late 3' UTR element (47). We observed that HIV-1 Rev and RRE or simian retrovirus type 1 CTE could reverse the effect of the inhibitory sequences in the L1 and L2 coding regions and induce high production of HPV-16 L1 protein from an L1 cDNA (Fig. 7) (19). Expression of HPV-16 L1 and L2 from recombinant Semliki forest virus, an alphavirus that replicates in the cytoplasm, resulted in high production of L1 and L2 (48), suggesting that the activity of the inhibitory L1 and L2 sequences required

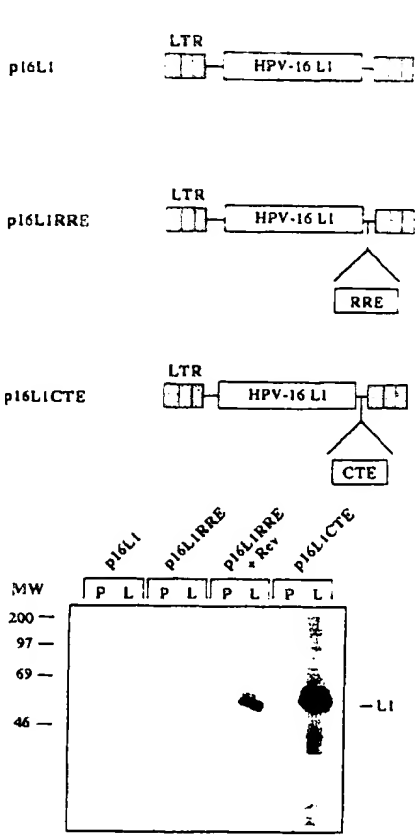


FIG. 7. Efficient expression of HPV-16 L1 using HIV-1 Rev and RRE or simian retrovirus type 1 CTE (46). (A) HPV-16 L1 expression plasmids. (B) Radioimmunoprecipitation of HPV-16 L1 produced in HeLa cells transfected with the indicated plasmids (19). P, preimmune serum; L, anti-HPV-16 L1 peptide antiserum.

nuclear factors. Taken together, these results indicate that Rev recognizes the RRE-containing mRNAs early in the mRNAs processing pathway, probably preventing interactions between the cellular *trans*-acting factors and the papillomavirus RNA inhibitory sequences by directing the RRE-containing mRNAs through an alternative mRNA processing and transport pathway. The effector domain of Rev interacts with a nucleoporin like protein and promotes export of RRE-contain-

ing mRNAs through a cellular protein export pathway (29, 32). Therefore, bypassing of certain steps in the nuclear mRNA processing pathway results in loss of inhibition, indicating that nuclear factors are required for inhibitory activity. However, HIV-1 Rev and RRE also affect the translation efficiencies of mRNAs (51, 52) and it cannot be excluded that HPV sequences interact with factors in the cytoplasm that affect mRNA utilization. Upon reflection, one may speculate that cellular factors similar in function to the HIV-1 Rev protein may be activated in terminally differentiated epithelial cells and induce production of the papillomavirus L1 and L2 capsid proteins. It is tempting to speculate that the HPV genome containing Rev and RRE or the CTE may be able to complete the entire replication cycle in nonterminally differentiated cells.

## ROLE OF THE INHIBITORY SEQUENCES IN THE PAPILLOMAVIRUS LIFE CYCLE

The presence of inhibitory sequences in both cutaneous (HPV-1) and genital (HPV-16) human papillomavirus types, as well as in bovine papillomaviruses (BPV-1), demonstrates that their presence is a conserved property of papillomaviruses and argues that these sequences play an important role in the papillomavirus life cycle. However, the sequence motifs encoding inhibitory activity vary among different papillomavirus types and their location can be either in the late 3′ UTR or in the late coding region. This may reflect the differences in tropism among the various papillomavirus types, i.e., preferential infection of epithelia of mucosal or cutaneous origin. Since the inhibitory HPV sequences are active in many different cell types and in cells from different species (17, 30), papillomaviruses apparently utilize a general cell regulatory machinery that presumably suppresses expression of various cellular genes, at least in many dividing cells or tumor cells. For example, the HPV-1 and the c-fos AU-rich elements interact with the same cellular factors (30) and act by reducing mRNA half life.

In contrast to HPV-16, the HPV-1 L1 and L2 coding regions lack sequences with strong inhibitory activity (60). Therefore, the presence of inhibitory sequences in the L1 and L2 coding regions appears to correlate with low production of virus *in vivo*, which is characteristic for HPV-16 (27), whereas negative elements are present in the 3′ UTR of HPV-1, HPV-16, and BPV-1, all of which display a differentiation-dependent late gene expression. Indeed, treatment of transfected epithelial cells with PMA, which has been shown to induce HPV-31 late gene expression in differentiated cells (33), relieved the block on gene expression caused by the HPV-16 late 3′ UTR negative element (38), suggesting that the inhibitory activity of the 3′ UTR element is alleviated in terminally differentiated cells. The reversal of inhibition may be a result of inactivation or down regulation of cellular factors interacting with the inhibitory sequences or perhaps a result of the presence of RNA binding proteins that reduce the effect of the inhibitory sequences, by binding either to these sequences or to distinct positive elements on the late mRNAs. Confinement of late gene expression to the superficial layers of the squamous epithelium may allow the virus to avoid the immune surveillance of the host. This would also ensure efficient spred of the virus since the terminally differentiated cells are destined to be shed into the environment.

## VIRUS INHIBITORY RNA SEQUENCES

HIV-1, human T-cell leukemia virus type 1 (HTLV-1), Mason-Pfizer monkey virus, simian retrovirus type 1, and hepatitis B contain well characterized mRNA sequences that interact with viral and cellular factors and regulate virus gene expression and virus production (54–59). Inhibitory sequences are located primarily on the mRNAs encoding structural proteins. These viruses all encode positive RNA elements that overcome the effect of the negative elements. *Cis*-acting RNA sequences with negative or positive effects on gene expression may provide viruses with a tool for fine tuned regulation of virus production which probably contributes to the ability of HIV-1, HTLV-1, hepatitis B, and papillomaviruses to persist in their hosts in the presence of specific immune responses against the viruses. One may speculate that RNA sequences that interact with cellular factors to downregulate the expression of virus structural proteins and virus production may be encoded by other viruses that establish persistent infections and that are not easily cleared by the immune responses of the host, e.g., hepatitis C and the herpesviruses.

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## RELATED PROCEEDINGS

There are no related appeal or interference proceedings.